# CLUSTER FORMATION IN PHA-STIMULATED MONONUCLEAR CELLS FROM PERIPHERAL BLOOD: EFFECTS OF COLCEMID AND TAXOL

## K. Sondell, C. Anderson, G. Holmgren, B.O. Norberg, I. Nordenson

Blood Center (KS), Departments of Clinical Genetics (CA, GH, IN), and Internal Medicine (BON), University Hospital, Umeå

## ABSTRACT

*Mononuclear cells from peripheral blood were incubated with phytohemagglutinin (PHA) for 24-72 hours. The cells formed dense cell clusters with firm cell-to-cell attachment and signs of cell communication, proliferation and differentiation. At the end of a 72-hour period of incubation, the test preparations were treated for 90 minutes with the classical microtubule antagonist Colcemid and the new microtubule antagonist taxol. Taxol produced approximately twice as many mitoses as Colcemid. Chromosomes in taxol-blocked mitoses appeared to be more contracted than chromosomes in Colcemid-blocked mitoses. It is suggested that one beneficial side effect of Colcemid preparation of chomosomes is stretching due to microtubule disassembly.*

Complementing the blood stream, the lymphatic system disperses fluid and cells to lymph nodes throughout the body. Cells, mainly lymphocytes and monocytes (macrophages) return from peripheral tissues to lymph nodes and exchange information on the immunologic "battlefield". This gathering of mononuclear cells with cell communication, proliferation, and differentiation constitutes an important function of lymphoid tissue and lymph nodes.

Cells involved in the immune response are readily collected from the bloodstream and subjected to *in vitro* experiments. Incubation of mononuclear leukocytes with phytohemagglutinin (PHA) is thought to stimulate T cell proliferation selectively (1-3). However, T cell proliferation is associated with differentiation in other coexisting cell lines. The aim of the present study is to describe cell interaction, proliferation, and differentiation in PHA-stimulated mononuclear cells from peripheral blood, comparing Colcemid and taxol as cytobiological tools.

## MATERIALS AND METHODS

### Blood sampling

Heparinized blood, 16 IU/ml, was obtained from two healthy women age 23 (experiments 5-7) and 35 (experiments 1-4) who donated the blood for all experiments described here (Tables 1,2).

### Isolation of mononuclear leukocytes

Mononuclear leukocytes (L-MNs) were isolated from peripheral blood by the one-step metriozoate-Ficoll procedure using Lymphoprep[R] (Nyegaard, Oslo, Norway). The L-MNs were then washed twice in standard medium and resuspended to a defined concentration.

## Table 1
## Cytocentrifuge Preparation of PHA–Stimulated
## Mononuclear Cells from Peripheral Blood

| | Incubation Time | |
| | 48 hours | 72 hours |
| --- | --- | --- |
| Experiment 1 | | |
| Cell density $(x10^5/ml)$ | 0.78 | 1.09 |
| Cell increase factor | | 1.4 |
| | | |
| Experiment 2 | | |
| Cell density $(x10^5/ml)$ | 0.62 | 1.22 |
| Cell increase factor | | 2.0 |

*Incubation*

L-MNs were incubated in 10ml glass tubes at 37ºC for up to 72 hours in the presence of PHA, final concentration 20µg/ml (Gibco Europe, Paisley, Scotland), in standard medium containing 400ml RPMI 1640 (Gibco), 100ml fetal calf serum, 10ml I-glutamine (Seromed, Berlin, FRG), corresponding to 100mM final concentration. Streptomycin and benzylpenicillin, 10µg/ml of each, were added. The medium was prepared fresh at the beginning of each experiment and was not changed thereafter.

*Cytocentrifuge preparation*

The cell suspension, 0.2ml, was layered above 0.1ml of standard medium in a cytocentrifuge (Shandon-Elliot Cytospin). The cells were then spun down on a slide for 10 min at 1,000rpm. Routine staining was by May-Grunwald-Giemsa. Cells were counted along a diameter of the slide preparation and converted to cells x $10^5/ml$ in the original cell suspension.

*Chromosome preparation*

Cells were spun down at 1,000rpm for 7 min, the supernatant evacuated, and cells hypotonically expanded by 0.075M KCl for 10 min. After re-centrifugation, the cells were fixed in acetic acid and ethanol in proportions 1:3. The fixation process was repeated three times with intercalcated centrifugations and evacuations of the supernatant.

*Chemicals*

Taxol (NCI, Bethesda, Maryland, USA) was dissolved in dimethylsulphoxide (DMSO, Sigma, St. Louis, Missouri, USA), 2mg/ml and stored at -20ºC. Prior to the experiment, taxol was diluted in phosphate buffer, pH 7.4, and added to the test solutions to final concentrations of 0.5 and 1.0µg/ml (6 and 12 x $10^{-7}$M). In the control preparations, DMSO was included (highest final concentration 0.05% v/v, Table 1).

Colcemid[R] (demecolcine) was obtained from Gibco Europe ready-made in Hank's solution, 10µg/ml. An opened vial was kept at +4ºC and discarded within a week.

Trypsin (Trypure[R], Novo, Baegsvaerd, Denmark) and collagenase (collagenase II, Sigma) were prepared in sterile solution.

All other chemicals used were of analytical grade.

*Statistics*

Student's paired t-test, two-tailed probabilities, was calculated by means of StatWorks[R] (Heyden & Son, London).

*RESULTS*

A starting cell density of 5 x $10^5$ cells per ml of cell medium appeared to

### Table 2
### Number of Mitoses Per 1000 cells in Different Hypotonic
### Preparations of PHA–Stimulated Mononuclear Blood Cells
### After Incubation for 72 Hours

|  | Control | DMSO | Colcemide | Taxol | Taxol |
|---|---|---|---|---|---|
| DMSO (%v/v) | – | 0.050 | – | 0.025 | 0.050 |
| Colc ($\mu$g/ml) | – | – | 0.1 | – | – |
| Taxol ($\mu$g/ml) | – | – | – | 0.5 | 1.0 |
| Experiment 1 |  |  | 46 | 39 | 61 |
| Experiment 2 |  |  | 35 | 49 | 56 |
| Experiment 3 |  |  | 28 | 32 | 46 |
| Experiment 4 | 11 | 10 | 41 | 44 | 73 |
| Experiment 5 | 19 | 16 | 38 | 63 | 74 |
| Experiment 6 | 20 | 12 | 41 | 55 | 75 |
| Experiment 7 | 17 | 12 | 47 | 69 | 75 |
| Mean |  |  | 39[a] | 50[b] | 66[c] |
| SEM |  |  | 3.5 | 5.0 | 4.4 |

Addition of test drugs 90 minutes prior to preparation
Statistical comparison: a–b (p=0.078), a–c (p<0.001), b–c (p=0.036)

be optimal. More cells (7–10 x $10^5$) produced crowded preparations with loss of cell detail. Fewer cells (2 x $10^5$) provided too few colonies for convenient analysis during the culture period chosen.

The morphological picture suggested a rapid cluster formation within the first 24 hours and then an intense proliferation *(Figs. 1,2)*. A rough estimate of cell proliferation was made from the cytocentrifuge preparations *(Table 1)*; the cell proliferation could not be quantified by counting in Burker chambers or automated equipment, since the cell colonies formed dense colonies simulating tissue fragments. These colonies could not be disaggregated into isolated cells by mechanical shaking and re–pipetting, nor by treatment with EDTA, trypsin or collagenase.

The cell colonies were distinguished by one or more central macrophage–like cells surrounded by small lymphocytes and blast cells in different stages of the cell cycle *(Figs. 1,2)*. Many lymphocyte–like cells displayed the hand–mirror shape typical of moving cells. A few plasmocyte–like cells were found scattered over the smear *(Figs. 1,2)*. This phenomenon suggested differentiation of some B cells

in these cell cultures.

Approximately 1–2% of the cells were in mitoses in control preparations after incubation for 72 hours. The taxol solvent DMSO did not affect the mitotic index. Addition of microtubule antagonists for 90 minutes increased the mitosis rate to 3–7%. Taxol treatment produced approximately twice as many mitoses as Colcemid treatment *(Table 2)*, but taxol–prepared chromosomes appeared more contracted than Colcemid–prepared chromosomes *(Fig. 3)*.

Cell kinetics derived from the above–mentioned mitotic indices suggested a duration of cell division of approximately 20 min and a cell cycle duration of approximately 20–40 hours. These calculations corresponded well with previous observations on cytocentrifuge preparations *(Table 1)*.

### DISCUSSION

Incubation of mononuclear leukocytes from peripheral blood with PHA produced dense cell colonies with appearance and properties akin to tissue fragments. Such cell clusters have provided the basic material for routine chromo-
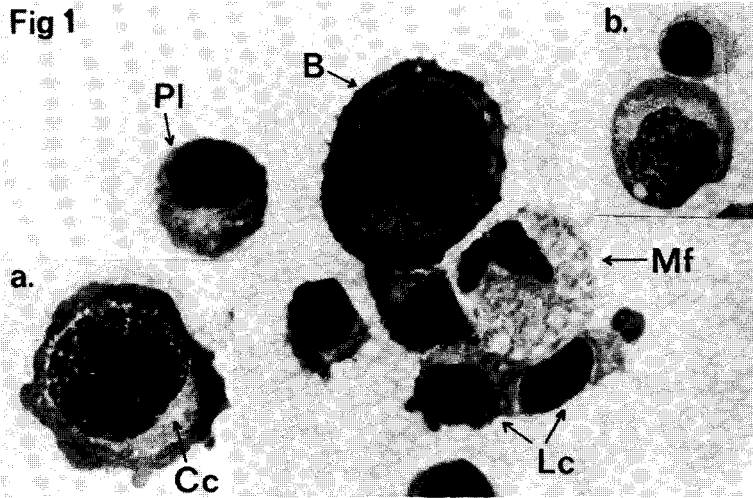
Fig. 1. The basic structure of a cell cluster in PHA-stimulated mononuclear cells from peripheral blood (main field). A central macrophage (Mf) with attached small lymphocytes (Lc). In the periphery of the cluster, a large blast cell (B) is approaching mitosis. A plasmocyte-like cell (Pl) is seen outside the cluster. Insets a. and b. show blast cells in prophase with a clear cytocentre (Cc), intact nuclear membrane, and visible chromosomes.
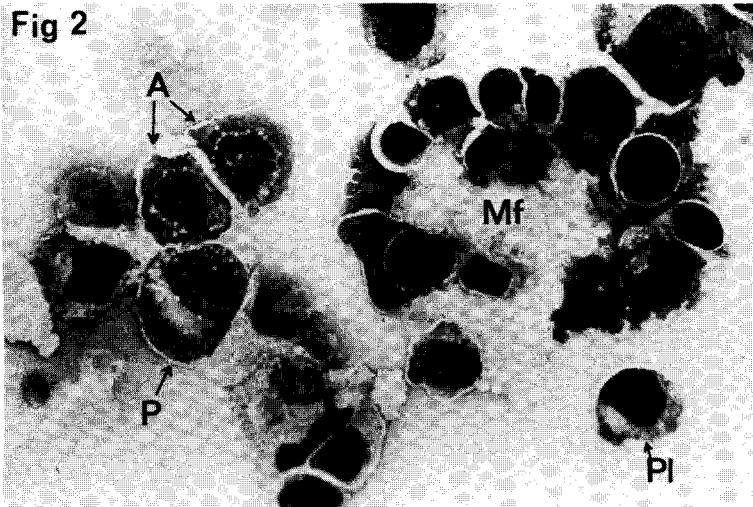


Fig. 2. A larger cluster with an out-floating central macrophage, surrounding small lymphocytes and peripheral blast cells in prophase (P) and anaphase (A). The anaphase daughter cells are separated by a non-stained midbody with the same MGG properties as the perinuclear cytocentre in the adjacent prophase cell.

some analysis since 1960 (4). Recent investigations have revealed an intricate interaction between ligand-receptor complexes and cell communication by lymphokines in these clusters (c.f. 1-3).

The main finding of the present study was that the new microtubule antagonist taxol produced approximately twice as many mitoses as the classical microtubule antagonist Colcemid (Table
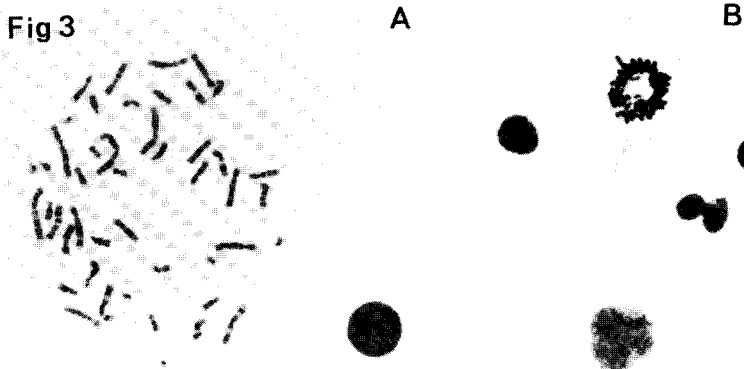
Fig. 3. *Hypotonic chromosome preparations, from metaphases blocked by Colcemid (A) and taxol (B).*

2). Both Colcemid and taxol concentrations were chosen to exceed precisely the threshold concentration of complete mitotic arrest, 0.1 and 1.0μg/ml respectively. It is evident from the difference between the two taxol concentrations that this goal was obtained.

Classical microtubule antagonists such as Colcemid and podophyllotoxin arrest cell division in a metaphase-like configuration due to microtubule disassembly. It has been, however, suggested from another model system of microtubule function that analogous end results may be obtained by the reverse process, that is, stabilization of microtubules (5).

The discovery of taxol, a complex ester isolated from the needles and bark of the Western yew, *Taxus brevifolia* (6), provides a complementary tool for the dissection of microtubule function in cell processes such as mitosis. In contrast to previous microtubule antagonists, taxol stabilizes microtubules by promotion of assembly (7-9), thus arresting cell division in the G2 and/or the M phase (10,11). Taxol is now undergoing therapeutic trial as an antineoplastic agent (6,12).

Our finding of twice as many mitoses in taxol preparations as in Colcemid preparations suggests that Colcemid exerts a degree of premitotic inhibition of the cell cycle. This interpretation is in agreement with a few previous reports on

pre-mitotic cell cycle effects of microtubule-disrupting agents (13,14). Conversely, taxol is thought to lack such pre-mitotic inhibitory effects on the cell cycle (c.f. 15).

Another interesting feature was that taxol-prepared mitoses appeared to provide more contracted chromosomes than Colcemid-prepared mitoses *(see Fig. 3)*. This observation is consistent with the idea that colchicine and other classical microtubule antagonists provide stretching of chromosomes due to microtubule disassembly (c.f. 16-18). This hypothesis explains the long-recognized favorable effect of colchicine treatment on chromosome morphology.

It is reasonable to assume that cell clusters formed by mononuclear blood cells in tissue culture provide a model of the immunological events of lymph cell communication, proliferation and differentiation (c.f. 19,20). The firm attachment of the cells in the cluster also produces an interesting basic material, a kind of microtissue fragments, for further investigation of microtubule function in dividing and differentiating cells.

*Note added in proof*

In a recent publication on tension generation by microtubule depolymerization using a new *in vitro* model, Koshland et al (Nature 131:504, 1988) accom-

panied by an editorial by R. Burns in the same issue support our contention that colchicine and like-agents improve chromosome morphology by inducing microtubule stretching.

## ACKNOWLEDGEMENTS

## REFERENCES

1. Isakov, N, W Scholtz. A Altman: Signal transduction and intracellular events in T-lymphocyte activation. Immunol. Today 9 (1986), 271.
2. Werdelin, O: Determinant protection. A hypothesis for the activity of immune response genes in the processing and presentation of antigens by macrophages. Scand. J. Immunol. 24 (1986), 625.
3. Piacibello, W, L Lu, D Williams, et al: Human gammer interferon enhances release from phythemagglutinin-stimulated T4+ lymphocytes of activities that stimulate colony formation by granulocyte-macrophage, erythroid, amd multipotential progenitor cells. Blood 68 (1986), 1339.
4. Therman, E: *Human Chromosomes. Structure, Behavior, Effects*, Ed. 2, Springer, New York, 1986.
5. Norberg, B: Cytoplasmic microtubules and radial-segmented nuclei (Rieder cells). Effects of osmolality, ionic strength, pH, penetrating non-electrolytes, griseofulvin, and a podophyllotoxin derivative. Scand. J. Haematol. 7 (1970), 349.
6. Wani, MC, HL Taylor, ME Wall: Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. J. Am. Chem. Soc. 93 (1971), 2325.
7. Schiff, PB, J Fant, SB Horwitz: Promotion of microtubule assembly *in vitro* by taxol. Nature (Lond.) 277 (1979), 665.
8. Manfredi, JJ, J Parness, SB Horwitz: Taxol binds to cellular microtubules. J. Cell. Biol. 94 (1982), 688.
9. Wallin, M, J Nordh, J Deinum: Changes in hydrodynamic properties of microtubules induced by taxol. Biochim. Biophys. Acta 880 (1986), 189.
10. Fuchs, DA, RK Johnson: Cytologic evidence that taxol, an antineoplastic agent from *Taxus brevifolia*, acts as a mititoc spindle poison. Cancer Treat. Rep. 62 (1978), 1219.
11. De Brabander, M, G Geuens, R Nuydens, et al: Taxol induces the assembly of free microtubules in living cells and blocks the organizing capacity of the centrosomes and kinetochores. Proc. Nat. Acad. Sci. USA 78 (1981), 5608.
12. Kris, MG, JP O'Connell, RJ Gralla, et al: Phase I trial of taxol given as a 3-hour infusion every 21 days. Cancer Treat. Rep. 70 (1986), 605.
13. Troupe, C: An *in vitro* study of the effect of cytostatic drugs on the DNA synthesis in experimental tumours. Diss. Lund, Sweden (1974).
14. Mauro, F, W Göhde, J Schumann, et al: Considerations in the design of possible cell cycle effective drugs. Int. J. Radiat. Biol. 49 (1986), 307.
15. De Brabander, M, G Geuens, R Nuydens, et al: Microtubule dynamics during the cell cycle: The effects of taxol and nocodazole on the microtubule system of Pt K2 cells at different stages of the mitotic cycle. Internat. Rev. Cytol. 101 (1986), 215.
16. Inoue, S, H Sato: Cell motility by labile association of molecules. The nature of mitotic spindle fibers and their role in chromosome movement. J. Gen. Physiol. 50 (1967), 259.
17. Malawista, SE, H Sato, KG Bensch: Vinblastine and griseofulvin reversibly disrupt the living mitotic spindle. Science 160 (1968), 770.
18. Norberg, B: Contractile processes in human lymphocytes and monocytes from peripheral blood. The oxalate-induced radial segmentation of the nuclei and the ATP-induced migration of glycerinated cell models. Scand. J. Haematol. Suppl. 14 (1971).
19. Donneberg, AD, J Cameron: Cinemicroscopic studies of lymphocyte behavior in semi-solid medium: The polyclonal origin of lymphocyte colonies. Exp. Hematol. 13 (1985), 29.
20. Bernheim, JL: DNA synthesis and proliferation of human lymphocytes. Diss. Vrije Univ., Bruxelles, Belgium (1978).

Bo Norberg, M.D.
Department of Internal Medicine
University Hospital
S-901 85 Umea, SWEDEN