drogue de dépolymériser les microtubules cytoplasmiques est une conséquence de son affinité envers les protéines microtubulaires[14,15]. Nos résultats indiquent en outre une scission des pôles du fuseau dans des cellules où la concentration de VLB n'atteint pas le niveau nécessaire à la complète destruction de l'appareil mitotique (blocage en métaphase).

La production d'anaphases multipolaires pendant le temps qui suit la cessation du traitement montre le progrès des cellules multipolaires au travers de la mitose; le retrait de la drogue permet à ces cellules d'échapper à la totale désorganisation de leur fuseau mitotique (c-métaphase).

Nous pensons que l'étude de la capacité multipolarisante de la VLB, après des traitements de courte durée, peut avoir de l'intérêt lors des applications pratiques de cette drogue dans la chimiothérapie anticancéreuse.

Summary. The effect of vinblastine on the cell anaphasic polarity in onion root meristems of *Allium cepa* L. has been studied. Multipolar anaphases production during recovery in tap water has been observed and its kinetic pattern established.

J. P. HERVÁS

Departamento de Citología, Facultad de Ciencias Universidad Autónoma de Barcelona, Bellaterra, Barcelona (Espagne), 2 August 1974.

[14] S. E. MALAWISTA, H. SATO and K. G. BENSCH, Science *160*, 770 (1968).

[15] R. MARANTZ, M. VENTILLA and M. SHELANSKI, Science *165*, 498 (1969).

Structure and Permeability of Junctions in Phytohemagglutinin Stimulated Human Lymphocytes

Specialized membrane junctions provide a direct pathway for intercellular communication in several tissues[1,2]. Gap-junctions have been found in several instances where electrotonic coupling was detected, but evidence indicating this structure as the site of low intercellular resistance is not conclusive[2]. An important correlation between ionic permeability and junctional structure has been described by GILULA et al.[3] in cultured Chinese hamster cells where gap junctions occurred only when high transjunctional permeability was also observed. More direct evidence has just been found by AZARNIA et al.[4] in a line of human cells where both intercellular communication and gap junctions were detected. When these cells were fused with a mouse cell line that has no electrotonic coupling and no gap junctions, a segregant non-coupling cell line was obtained, in which no gap-junctions were detected.

Human lymphocytes stimulated by the plantderived mitogen phytohemagglutinin (PHA) form junctions of high permeability[5]. Increase of cell density enhances the mitotic activity induced by PHA-stimulation[6], a

[1] W. R. LOEWENSTEIN, Ann. N.Y. Acad. Sci. *137*, 441 (1966).

[2] M. V. L. BENNETT, Fedn. Proc. *32*, 65 (1973).

[3] N. GILULA, R. REEVES and A. STEINBACH, Nature, Lond. *235*, 262 (1972).

[4] R. AZARNIA, W. J. LARSEN and W. R. LOEWENSTEIN, Proc. natn. Acad. Sci., USA *71*, 880 (1974).

[5] G. M. OLIVEIRA-CASTRO, M. A. BARCINSKI and S. CUKIERMAN, J. Immun. *111*, 1616 (1973).

[6] J. H. PETERS, in *Lymphocyte Recognition and Effector Mechanisms* (Eds. K. LINDAHL-KIESSLING and D. OSOBA; Academic Press, New York 1974), p. 13.

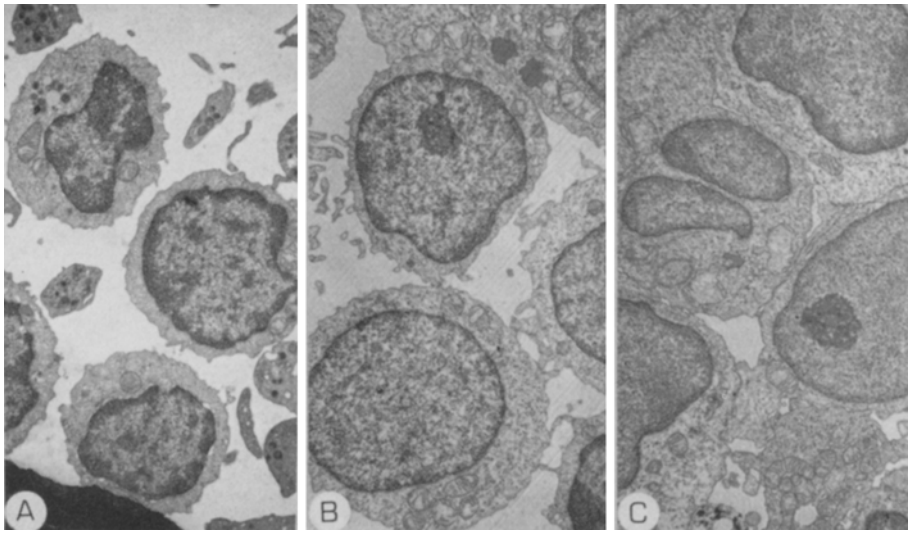

Fig. 1. Lymphocyte cultures after different periods of stimulation by PHA. ×3,200. A) Control culture after 24 h of incubation without PHA. Note that no cell contacts are seen. B) Culture after 24 h of incubation with PHA. Several contacts between the stimulated cells are present. C) Culture after 65 h of incubation with PHA. All the cells are in contact and the culture has a tissue-like appearance.

Time in culture (h)	Coupled cell pairs (%)	V_2/V_1 [a]		No. of pairs tested
		X ± S.D.	Range	
1	14,3	0.18 ± 0.08	0.09–0.30	14
24	45,5	0.38 ± 0.06	0.17–0.45	11
48	75	0.42 ± 0.05	0.29–0.51	16
65	80	0.41 ± 0.07	0.30–0.56	10

[a] Coupling coefficient.

membrane mediated function of the mitogen. In the present investigation we have studied the development of intercellular communication in PHA-treated human lymphocytes by electrophysiological measurements of permeability and concomitant ultrastructural analysis.

Material and methods. Peripheral blood of healthy human donors was separated on a Ficoll-Hipaque gradient and cultures were prepared according to the technique described previously[5]. The electrophysiological measurements were performed in Roswell Park Memorial Institute Medium as described elsewhere[5].

Glass microelectrodes filled with 3 *M* KCl and 2 m*M* K-citrate (resistances of 35–65 megΩ) were manipulated into 2 neighboring cells. Electrode 1 was used to pass hiperpolarizing current pulses between the cytoplasm of one cell and the extracellular fluid. It was connected to a bridge-like preamplifier allowing simultaneous monitoring of the voltage drop (V_1) caused by the injected current as it crossed the cell membrane. Electrode 2 was used to monitor the voltage drop (V_2) caused by the current pulse in an adjacent cell. The ratio of V_2/V_1 is an indication of the transjunctional conductance. Rectangular current pulses of 10^{-8} to 10^{-9} A and 50 msec duration were used. Current injection and recording was monitored with a standard electrophysiological set-up (see [5] for details).

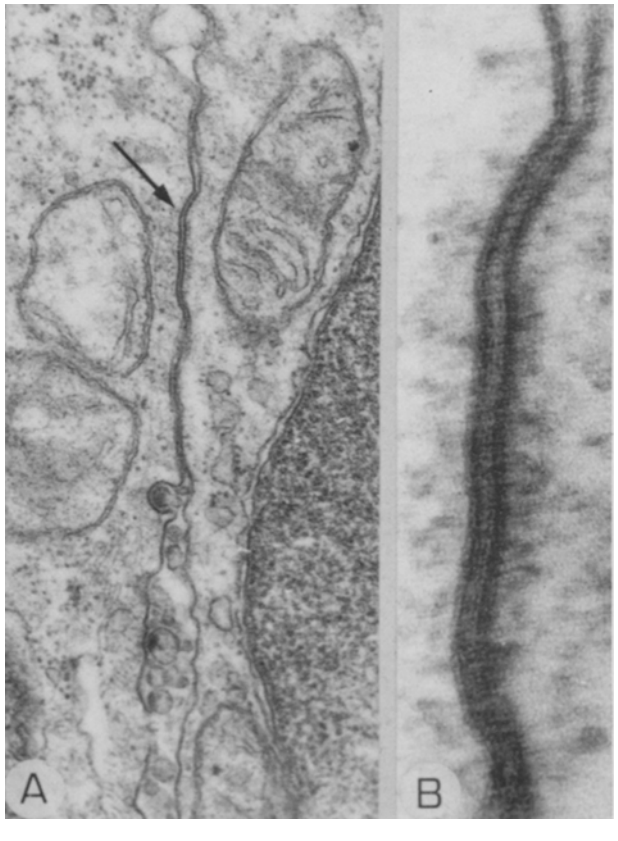

Fig. 2. Junctions between stimulated lymphocytes after 65 h of incubation with PHA. A) Portion of the cell junction. ×30,000. B) Higher magnification of Figure A (arrow). Note the appearance of the junctional complex. ×200,000.

Cells cultured for 1, 24 or 65 h with and without PHA were fixed and the ultrastructure of their junctions analyzed. The best fixation was obtained by slight modification of the method of HIRSCH and FEDORKO[7]. Immediately before use, 2 volumes of 2.5% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.4) were added to one volume of 1% osmium tetroxide, at 0 °C. The fixative was gently added to the lymphocytes after removal of the culture medium and replaced after one hour with Earle's salt solution. The cell suspension was then stained with 0.25% uranyl acetate in 0.1 *M* acetate buffer (pH 6.3). Constant temperature (0 °C) was maintained throughout the fixation and staining procedure. To preserve the original cell density and avoid distortions of the clumps, centrifugation was never used. After embedding in EPON mixture the cells were sectioned with an Ultratom III (LKB Products). Thin sections showing silver interference colours were stained with 0.4% lead citrate and examined with an EM6-B electron microscope (A.E.I.).

Results and discussion. Transjunctional conductance was detected after 1 h of PHA treatment. Earlier measurements did not yield reliable results due mainly to frequent cell disaggregation consequent to impalement. An increase in coupling coefficient was observed simultaneously with the increase in intercellular contacts. The mean value of the coupling coefficient reaches a maximum at 48 h, as can be seen in the Table.

The number of cells establishing contact increased with the incubation period as shown if Figure 1. In control cultures, cultived in the absence of PHA, no cell contacts could be observed (Figure 1A). Cultures submitted to the same incubation period (24 h) with PHA show several areas of contact between the stimulated cells (Figure 1B). After 48 to 65 h, the cells are densily packed and form a tissue-like structure (Figure 1C).

Thin sections of stimulated lymphocytes show local differentiations of the plasma membrane in the zones of intercellular contact. These contacts were seen after 1 h of PHA-stimulation mostly in pseudopodlike processes.

In the areas of close contact the plasma membranes run strictly paralel. The membrane outer leaflets which are separated by a gap of approximately 30 Å are less intensively stained than the cytoplasmic leaflets and can sometimes be visualized as a dotted line (Figure 2). The average thickness of the junctional complex is approximately 200 Å.

[7] J. G. HIRSCH and M. E. FEDORKO, J. Cell Biol. *38*, 615 (1968).

The 2 outer electron-dense lines have an average thickness of 60–70 Å. The total length of the intercellular contact increased with the incubation period and after 48 h the cell contacts covered almost the entire membrane area.

If we consider the coupling ratio (V_2/V_1) as a convenient index of the junctional membrane permeability, its increase as a function of the PHA incubation period suggests a concomitant increase in the low-resistance structures responsable for the intercellular permeability. Since in our preparations the areas of close cellular contact broadened with the incubation time, and the only specialized junctional structures which appeared were identified as gap junctions, we have inferred that these structures are identical with the low-resistance pathways in stimulated lymphocytes. Our identification of the contacts as gap junctions is based on: a) the thickness of the junctional complex (200 Å), which is greater than that of the zonula occludens (about 140–150 Å); b) the periodicity of the electron-dense particles of the median line of the junctional profile, which corresponds to the centre-to-centre spacing of the freeze-etch particles previously described in gap junctions[8–10]; c) gaps of about 30 Å between the two adjacent cell membranes.

The exact mechanism involved in lymphocyte cell stimulation is not known, but it is widely accepted that the membrane is the site where the mitogen acts. The dependence of lymphocyte stimulation by PHA on cell density and on cell-to-cell contact, and the appearance of intercellular communication in human and bovine lymphocytes after 12 min of PHA treatment[11], suggest a functional role for the low-resistance pathways. Since cellular associations have been observed to occur both in vivo and in vitro in conjunction with the expression of several immune responses, we suggest that lymphocyte-lymphocyte cooperation through gap junctions provide a model system for the study of such cellular interactions. Lymphocyte junctions have some characteristics in common with those described for epithelial cells. An increase in cytoplasmic Ca^{++} concentration causes a marked reduction of the junctional conductance[12] in a way similar to that previously described for salivary gland cell junctions[13].

In a number of different cell systems where intercellular communication has been detected, it has not been possible to establish a correlation between specific morphological structures and the physiological evidence of cellular communication, because of the coexistence of several different types of specialized junctional complexes. As in several other preparations[4,8,10], in human lymphocytes stimulated by PHA only gap junctions have been found. We suggest that these structures are the low resistance channels responsable for intercellular communication in the present cell system. Studies with extracellular space markers are required for a more definitive identification of these junctional complexes and we are at present working on that[14,15].

Résumé. La microscopie électronique et une méthode électrophysiologique ont été utilisées pour étudier les jonctions entre les lymphocytes humains stimulés par la phytohémaglutinine. Le coefficient de couplage électrotonique augmente avec le temps d'incubation. Les seules intéractions cellulaires typiques mises en évidence par la microscopie électronique sont les «gap jonctions».

I. F. GAZIRI[16], G. M. OLIVEIRA-CASTRO[17], R. D. MACHADO[18] and M. A. BARCINSKI

Instituto de Biofísica, Universidade Federa do Rio de Janeiro, Cidade Universitaria, Rio de Janeiro GB, Zc-32 (Brazil 20.000), 16 August 1974.

[8] P. PINTO DA SILVA and N. B. GILULA, Expl. Cell Res. *71*, 393 (1972).
[9] D. A. GOODENOUGH and J. P. REVEL, J. Cell Biol. *45*, 272 (1970).
[10] D. F. HÜLSER and A. DEMPSEY, Z. Naturforsch. *28*, 603 (1973).
[11] D. F. HÜLSER and J. H. PETERS, Expl Cell Res. *74*, 319 (1972).
[12] G. M. OLIVEIRA-CASTRO and M. A. BARCINSKI, Biochim. biophys. Acta *352*, 338 (1974).
[13] G. M. OLIVEIRA-CASTRO and W. R. LOEWENSTEIN, J. Membrane Biol. *5*, 51 (1971).
[14] Acknowledgment: We thank Dr. MARTHA SORENSON for helpful discussion.
[15] Supported by grants from Conselho Nacional de Pesquisas (CNPq), Banco Nacional de Desenvolvimento Econômico (FUNTEC-241), Conselho de Ensino para Graduados da UFRJ (CEPG-UFRJ).
[16] Fellow of the Conselho Nacional de Pesquisas. Present address: Departamento de Fisiologia, Universidade Federal de Pernambuco, Recife, PE, Brasil.
[17] Correspondence to: G. M. Oliveira-Castro, Universidade Federal do Rio de Janeiro, Instituto de Biofísica, Cidade Universitária, Rio de Janeiro, GB, ZC 32-20.000, Brasil.
[18] Fellow of the Conselho Nacional de Pesquisas.

Seasonal Variations in the Fertilizing Capacity of *Bufo arenarum* (Amphibia Anura) Spermatozoa[1]

Artificial fertilization of *Bufo arenarum* oocytes can be routinely obtained throughout the year. This species has a continuous spermatogenic cycle with seasonal fluctuations[2]. Testes size changes considerably during the year, maximal size being observed at the beginning of the breeding season, in the spring. During the winter season, when testes are increasing their size, sperm clusters are observed adhered to Sertoli's cells in the seminiferous ampullae[3]. It is likely that spermatozoa remain in the testes until spring, since they have not been observed in urine samples obtained in winter[4].

The fertilizing ability of amphibian spermatozoa, as far as we know, has not been explored and is the subject of this paper. We also studied the decline of the fertile life of spermatozoa incubated in salt solution both in summer and winter season. A further objective was to establish whether soluble products removed from the testes had any influence on the fertilizing capacity of spermatozoa incubated in it.

[1] This work was supported by a grant from the Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina and by the Population Council grant No. 71.68 awarded to the Consejo Nacional de Investigaciones Científicas y Técnicas to be administered by the Instituto de Biología, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán.
[2] M. H. BURGOS and R. E. MANCINI, Revta Soc. argent. Biol. *24*, 328 (1948).
[3] M. H. BURGOS and R. E. MANCINI, Revta Soc. argent. Biol. *23*, 154 (1947).
[4] M. H. BURGOS and R. E. MANGINI, Revta Soc. argent. Biol. *23*, 165 (1947).