

## CONTACT COOPERATION IN STIMULATED LYMPHOCYTES

### II. *Electrophysiological Investigations on Intercellular Communication*

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#### SUMMARY

Intercellular communication—as defined by the flow of ions between cells through low-resistance junctions—exists in agglutinates of phytohemagglutinin-stimulated lymphocytes. It is shown that this communication starts within minutes after the addition of the stimulant, at the same time as other functions of the lymphocyte membrane are altered. In contrast, neither stimulation nor intercellular communication can be detected under conditions where agglutination was established by a lymphocyte-agglutinating anti-serum, indicating that communication may be closely correlated with the process of stimulation.

Intercellular communication, as measured by electrical ion flow between non-excitabile mammalian cells, is a widespread phenomenon in organized tissues [4, 6, 19, 22, 27] as well as in cultured cells of different origin [5, 6, 12, 20]. It has been shown that when electrical coupling occurs, an intercellular exchange of substances can also be demonstrated using dyes of molecular weight up to 1 000 [6, 13, 27, 33]. This has been interpreted as an indication of information exchange between these communicating cells [6, 15, 16].

Many experiments have been performed to investigate whether intercellular communication can be influenced by changing extra- and intracellular conditions [18, 26]. These studies have been mostly concerned with the underlying mechanism of intercellular communication. Another approach is the measurement of communication between cells which can be induced to change from one physiological

state to another [10, 11, 14, 27]. These prerequisites are fulfilled by small lymphocytes which can be triggered by non-specific as well as immunologically specific stimulants to shift from a resting to an activated state with high metabolic activity. The unspecific stimulation (e.g. with phytohemagglutinin) induces a higher percentage of cells and is therefore a favorite model for biochemical and cell-physiological studies of this activation process. Interestingly, this stimulation is accompanied by cell agglutination. As has been described in a previous paper [23], cell contact, which may be enhanced by this agglutination, is a prerequisite for stimulation. In this paper we show that the onset of phytohemagglutinin stimulation is paralleled by the establishment of intercellular communication within a lymphocyte agglutinate.

The small diameter of lymphocytes (10  $\mu\text{m}$ ) made microelectrode measurements more

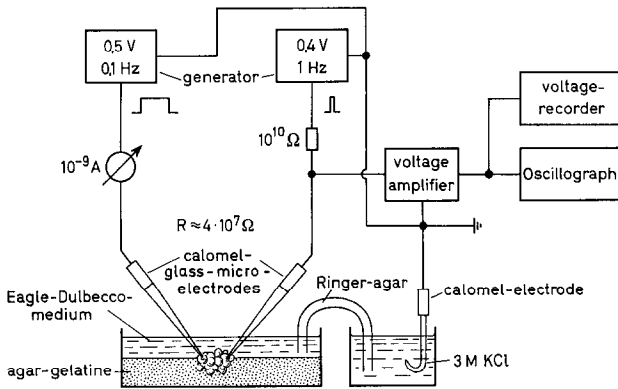


Fig. 1. Scheme of the electrical set-up: The 'current electrode' (left microelectrode) was supplied with 0.1 Hz rectangular pulses of about  $2-3 \times 10^{-8}$  A. The resulting voltage changes were recorded by the 'recording electrode' (right microelectrode) together with 1 Hz pulses of the resistance measurements. A Ringer-agar bridge connected the medium to a 3 M KCl solution and to the indifferent calomel electrode. Lymphocytes were embedded in an agar-gelatine mixture; insertions of the electrodes were controlled by phase-contrast observation (for details see text).

difficult, impalement being possible only after modification of embedding and observation methods.

## MATERIALS AND METHODS

### Lymphocytes

Lymphocytes were obtained from bovine lymph nodes, purified by passage through a glass fibre column and cultivated as described previously [7]. This procedure results in a more than 95% pure lymphocyte preparation. The impurity is due to contamination with reticulum cells; macrophages could not be detected by May-Grünwald-Giemsa staining of smears. Lymphocytes were stimulated with phytohemagglutinin-P (Difco) and pulse-labelled with  $^{14}\text{C}$ -uridine under standard conditions as described previously [7, 23]. Lymphocyte agglutination without stimulation was performed by use of a 10% horse-anti-pig-thymocyte serum.

For microelectrode measurements the cells had to be immobilized. For this purpose, the bottom of a plastic Petri dish (50 mm diameter; Greiner, Nürtingen, Germany) was scraped with a steel needle in order to obtain a better attachment of the agar film. This film was prepared in the following way: A stock solution of 2% Agar (Agar Noble Special; Difco) in 0.9% NaCl was heated at  $100^\circ\text{C}$ . During the cooling period, 0.8 ml were mixed with 1.2 ml of a  $37^\circ\text{C}$  solution of Eagle medium, supplemented with 10% inactivated calf serum and 0.6% gelatine (Merck, Germany). At  $37^\circ\text{C}$   $2 \times 10^7$  sedimented lymphocytes were suspended in 0.15 ml of this solution, pipetted into the Petri dish and smoothed down with a glass needle. After a gelatinization period of 5 min at room temperature 2.5 ml Eagle medium + 10% calf serum was poured onto the agar layer. Viability of these immobilized cells was confirmed by the trypan-blue exclusion test. Trypan-blue positive cells are identical with those which, under phase-contrast observation, appear as dark cells as compared with the brightly appearing viable lymphocytes. Usually less than 10% of the cells were trypan-blue positive, a ratio which

is similar to that observed under normal cell-culture conditions.

### Electrical measurements

The principle of the electrical measurements can be seen in fig. 1. We used two Ling-Gerard glass microelectrodes with especially thin tips of less than  $0.5 \mu\text{m}$  diameter (resistances  $\geq 40 \text{ M}\Omega$ , tip potentials  $\leq 5 \text{ mV}$ ) to measure the communication between lymphocytes. Both electrodes were held by a plexi-glass holder and were connected through a 3 M KCl solution to calomel electrodes.

The 'current electrode' (left microelectrode, fig. 1) was connected to a modified Tektronix generator (Tektronix 601/161/162) via a nano-ampere-meter (Philips digital multimeter PM 2421) and was supplied with 0.1 Hz rectangular pulses of about  $2-3 \times 10^{-8}$  A. This current flows from the electrode through the medium and a glass bridge filled with Ringer-agar to the indifferent calomel electrode (right in fig. 1). The successful impalement of a cell by the current electrode was indicated by the decreased current due to the high ohmic resistance of the intact lymphocytes.

The 'recording electrode' (right microelectrode, fig. 1) was connected to a Keithley amplifier (Keithley electrometer, Mod. 605) and via a  $10^{10}\Omega$  resistor 1 Hz pulses were supplied from a second Tektronix generator. These pulses were used for continuous resistance control [8], since the voltage change measured with the amplifier depended on the electrode resistance plus—in the case of impalement—the ohmic resistance of the cell. Successful impalement of the cells with the recording electrode was indicated by a potential difference and an increased pulse height of the resistance measurements and—in the case of communication—by a periodical voltage change corresponding to the 0.1 Hz pulses of the current electrode. These measurements were recorded with a voltage pen-recorder (Graphirac, Sefram). For further details see [9].

The impalements were made at room temperature under microscopical observation (Zeiss Standard RA) with a water-immersion objective ( $40\times$ ) which was electrically insulated by coating the metallic conus with a beeswax-colophonium mixture which had no demonstrable toxic effect on the cell cultures during

the measurements. This procedure allowed the observation of lymphocytes at a 400 times magnification, which was necessary, since lymphocytes are among the smallest mammalian cells.

## RESULTS

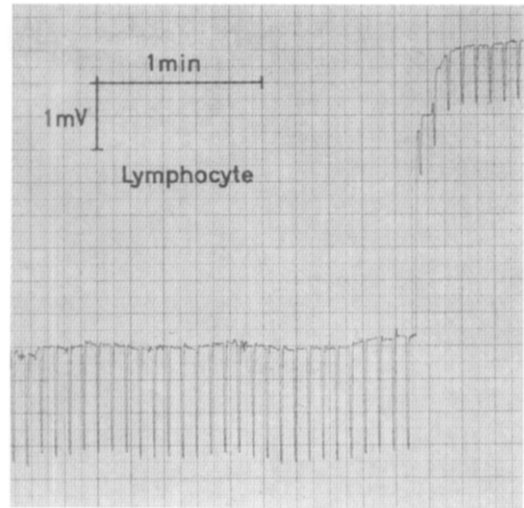
In early experiments, a bovine fibrin film was used to embed the lymphocytes according to a method described by Schindler [31] which was expected to be the most physiological way of cell immobilization. However, when an electrode was moved through the fibrin network, this was dragged in such a manner that neighbouring cells were damaged. This effect did not occur with the described agar-gelatin embedding.

A successful insertion of the electrode into a lymphocyte did not change the appearance of the cell under phase-contrast observation. This means that the cell membrane had sealed closely around the electrode shank. The microscopical appearance of the cell was another parameter for cell viability after insertion of the electrode, in addition to the electrical parameters described before.

### *Membrane potentials*

After insertion of the electrode into a lymphocyte, the transmembrane potential difference was 10 mV or less and remained stable for up to 1 h. This potential difference was either negative, as one would have expected from measurements on other mammalian cells, or positive. A positive potential difference was mainly found when the tip potentials of the electrodes were close to  $-5$  mV. The most important factor influencing the potential-difference measurements may be this individual tip potential of different electrodes, which is relatively high as compared with the generally low transmembrane potential of lymphocytes.

Addition of phytohemagglutinin to resting lymphocytes during the measurements caused



*Fig. 2.* Resistance measurement of a lymphocyte membrane. (*Left*) the situation of an impaled lymphocyte. The pulse heights indicate the ohmic resistance of the electrode plus the cell membrane. After withdrawal of the electrode the potential line returns to the base value and the pulse heights decrease, representing only the electrode resistance. From the known electrode resistance (in this case  $40\text{ M}\Omega$ ) and an estimated lymphocyte surface area of about  $300\ \mu\text{m}^2$  a specific membrane resistance of about  $125\ \Omega\text{cm}^2$  is calculated.

only insignificant changes of the measured potential differences. Measurements at different times up to 48 h after phytohemagglutinin stimulation also showed no significant difference as compared with non-stimulated control cells.

### *Membrane resistances*

Measurement of membrane resistance by the described method is mainly influenced by two parameters: one is the specific membrane resistance, the other is the extent of the surface area. This means that with identical specific membrane resistance a smaller cell has a higher ohmic resistance than a larger cell. The small size of lymphocytes, therefore, facilitated the resistance measurements with this method. In *fig. 2* the higher pulses indicate the membrane resistance plus electrode resistance. After withdrawal of the electrode

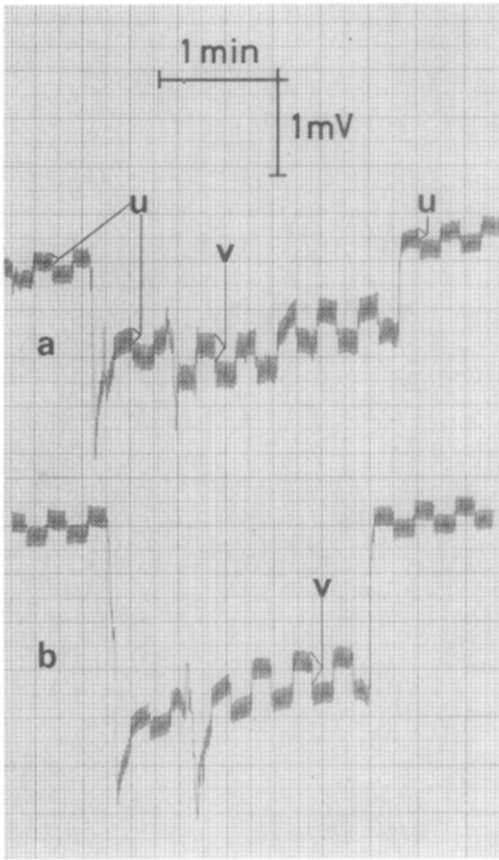


Fig. 3. Intercellular communication between phytohemagglutinin-agglutinated lymphocytes. Two (*a*, *b*) independent measurements in different agglutinates. The potential difference is measured by insertion of the recording electrode into a lymphocyte. The communication between the cells can be seen by the increased coupling pulses after insertion of the current electrode (for details see text).

only the electrode resistance pulses are registered. The different height of the potential line gives the membrane potential value. The ohmic resistance of the lymphocyte membrane is of the same order as the electrode resistance, since the pulse heights increase up to twice the value. Estimation of the specific membrane resistance of lymphocytes results in a value of more than  $100 \Omega \text{cm}^2$ . This value is of the same order as in other mammalian cells [5]. As in the measurements

of the potential difference, no significant changes of the membrane resistance could be detected after the addition of phytohemagglutinin.

The pulse indicating the membrane resistance of cells seemed to be less within a large agglutinate than in smaller agglutinates or in single cells. This may be due to the fact that the surface area influencing the resistance measurements is increased by the establishment of low-resistance junctions between communicating cells. However, these findings should not be stressed too much.

#### *Intercellular communication*

In order to investigate intercellular communication, two microelectrodes were used, one for recording the membrane potential, and another one for injecting current pulses into cells nearby. In control experiments using non-agglutinated and non-stimulated lymphocytes, no intercellular communication could be detected. When the electrodes had been inserted into adjacent cells, the pulses observed with the recording electrode during current flow through the current electrode ("coupling pulses") did not exceed the pulses which were already observed with both electrodes situated in the bath. These "bath coupling pulses" arise from the electrical circuitry and to a small extent from the resistivity of the medium.

In agglutinates of phytohemagglutinin-stimulated lymphocytes communication could be measured not only between adjacent cells but even over a distance of about 10 cells. In fig. 3 the recordings from two experiments (*a*, *b*) can be seen, showing the communication which occurred between lymphocytes separated by a different number of cells. Starting on the left side, one can see the bath coupling pulses (*u*, 0.1 Hz) as well as the pulses monitoring the electrode resistance (1 Hz) which are superimposed onto the potential base

line. Insertion of the recording electrode led to a sudden change of the potential line which was followed by a transient drift of the potential difference to a lower value. The bath coupling pulses ( $u$ ) remained constant after insertion since the current electrode was still in the bath and only the pulses of the resistance measurements increased. Half a minute later the current electrode was inserted into another cell of the same agglutinate. This caused a short voltage deflection and the establishment of the coupling pulses ( $v$ ), indicating communication between the impaled cells. After withdrawal of the recording electrode, the potential returned to the base line value and the coupling pulses decreased at the same time to the level of the bath coupling.

The maximum pulse height ( $V_1 = v_1 - u$ ) obtained by insertion of both electrodes into one cell can be compared with the pulse heights ( $V_2 = v_2 - u$ ) obtained by insertion of both electrodes into different cells. The 'communication ratio'  $V_2/V_1$  [17] is an indicator for the extent of intercellular coupling. In our experiments it was not possible, routinely, to determine  $V_1$  for each measurement. Therefore, only estimates for the communication ratio can be given: For adjacent cells a maximum value of about 0.5, and for more distant cells of about 0.1, can be estimated.

In further experiments, it was investigated whether intercellular communication can also be seen in agglutinated lymphocytes which are not stimulated by the agglutinating agent. It was found that a horse-anti-pig-thymocyte serum agglutinated bovine lymphocytes without stimulating them (see also [29, 30]). Fig. 4 shows that there is only negligible stimulation of uridine incorporation into anti-serum treated cells as compared with calf-serum incubated controls. Furthermore, there appears to be no toxic effect of this serum, since lymphocytes preincubated

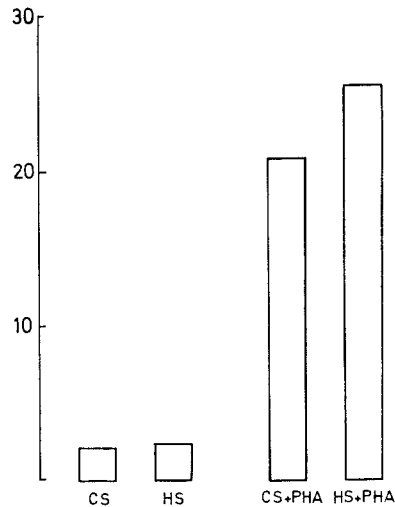


Fig. 4. Ordinate: pMoles/7.5 · 10<sup>6</sup> cells/h. CS, Control cultures with 10% calf serum. HS, cultures 24 h with horse serum. CS + PHA, 24 h preincubated with 10% calf serum; 24 h incubated with 10% calf serum + phytohemagglutinin. HS + PHA, 24 h preincubated with 10% horse serum; 24 h incubated with 10% horse serum + phytohemagglutinin.

Influence of horse-anti-pig-thymocyte serum on uridine incorporation into bovine lymphocytes. Cells were preincubated under different conditions and tested for <sup>14</sup>C-uridine incorporation (0.05 μl/1.5 ml [62 mCi/mMoles]) for 1 h into acid-insoluble material.

for 24 h with anti-serum can be easily stimulated with phytohemagglutinin. In addition, the trypan-blue exclusion test indicated no toxic effect. In communication measurements between these anti-serum agglutinated cells no electrical coupling could be found even in adjacent cells. This shows that intercellular communication is not necessarily linked to agglutination.

This agglutination system also enabled us to observe the initiation of intercellular communication after the addition of phytohemagglutinin. Two adjacent cells of an anti-serum mediated lymphocyte agglutinate were impaled and coupling pulses registered identical to the bath coupling pulses, remaining unaltered for up to 1 h. When phytohemagglutinin was added during such a measurement, the onset of intercellular communica-

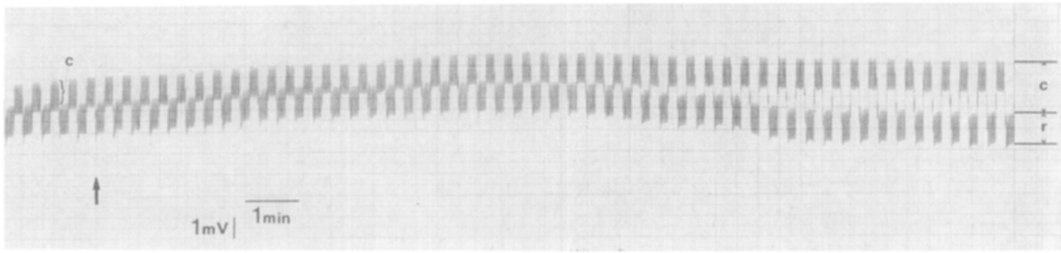


Fig. 5. Onset of intercellular communication between lymphocytes after addition of phytohemagglutinin. Electrodes were inserted into two lymphocytes of a non-stimulated agglutinate prior to the beginning of the tracing. The coupling pulses recorded in this situation (*left*) do not exceed the bath coupling pulses. The arrow indicates the moment when phytohemagglutinin was added to the culture. After about 7 min the communication started, as can be seen by the increasing pulse *c*, and was fully established after about 12 min. Pulse *r* indicates the ohmic resistance of the system (electrode + lymphocyte).

tion could be observed. Fig. 5 shows the recording of such an experiment. The arrow indicates the time of phytohemagglutinin addition. In this case communication started after 7 min and was fully established after about 12 min.

In different experiments different intervals between phytohemagglutinin addition and onset of communication were seen, the shortest being one minute [10, 11]. This interval seemed to depend on the diffusion time of phytohemagglutinin. When the impaled cells were on top of the agar and the stimulant was added very close to these cells, the communication started within a shorter time.

## DISCUSSION

Intercellular communication has been detected in many tissues and may indicate an exchange of signals between cells. The nature of this hypothetical information, however, is unknown. In addition, until now no mammalian system has been found where changes of the intercellular communication could be correlated with an alteration of the physiological state of the cells. In this respect, the lymphocytes are an important model, since the beginning of stimulation [24] occurs at the same time as the onset of communication.

The coincidence of these events must not

necessarily involve a functional connection. We have tried experimentally to separate the three phenomena: agglutination, stimulation, and intercellular communication. Agglutination could be produced without accompanying stimulation and communication, this indicating that close cell contact does not necessarily involve communication. This was also shown by manipulating non-stimulated cells into close contact. When, on the other hand, agglutination was accompanied by stimulation, intercellular communication was detected. This may be due to different sites at the cell surface: unspecific sites where the cells can be bound together without triggering other events, and more specific sites where an agglutination of cells is associated with stimulation and intercellular communication. These specific sites react within minutes of addition of phytohemagglutinin, simultaneously producing agglutination, intercellular communication and, as an early sign of stimulation, an increase of transport processes [24, 25, 28].

Similar results have been described by Loewenstein [14] for a completely different cell system: Separated sponge cells reaggregate only with cells of the same species, indicating that specific sites at the membrane exist for recognition. Only between reaggregated cells of the same species communication

could be found. This means that the establishment of communication is closely connected with the recognition process.

Although the mechanism of lymphocyte stimulation is not completely understood, it is known that phytohemagglutinin as well as other plant-originating stimulants soon adhere to the cell membrane when added to lymphocyte cultures [1, 34]. It has been shown previously that the first reactions of lymphocytes to the stimulant are connected with the cell membrane. Immediately after addition of phytohemagglutinin the transport capacity of the cell membrane increases considerably, as has been shown for uridine [24], glucose [25], amino acids [3] and ions [2, 28]. The rate of <sup>14</sup>C-uridine incorporation into lymphocyte RNA is controlled by the rate of transport across the cell membrane [24].

These early events indicate that the cell membrane may be the site where the first steps of lymphocyte stimulation are controlled. The establishment of intercellular communication after addition of phytohemagglutinin to lymphocytes is probably not just another early reaction of the activated lymphocyte membrane, but may also have a function as a mediator for cellular cooperation during the process of stimulation. Taking into account the results presented in the previous paper [23], lymphocyte stimulation could, therefore, be interpreted as a multicellular process rather than a single-cell phenomenon, since it appears to be dependent on cellular interaction.

This cellular cooperation must not necessarily require a specific information exchange between cells, as has been discussed in the previous paper. However, in specific immune reactions, interactions between certain different cell types are necessary (for a review see [21]). Furthermore, Sellin et al. [32] have recently observed the presence of intercellular communication in specific immune reactions

in the form of a flow of fluorescein from one cell to another. These analogies between specific and unspecific lymphocyte stimulation indicate again that the unspecific lymphocyte stimulation may be a useful model for the study of the mechanism of lymphocyte cooperation.

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