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Cyclosporin A Inhibits Long-Term Activation of Na^+,K^+ Pump in Phytohemagglutinin-Stimulated Human Lymphocytes

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The effect of the immunosuppressing drug cyclosporin A (CsA) on the K (Rb) influx, intracellular K and Na contents, and on the major parameters of lymphocyte activation have been investigated in human peripheral blood lymphocytes activated by phytohemagglutinin (PHA). CsA suppressed protein, RNA, DNA syntheses and cell proliferation by 49.8±4.3, 67.6±10.1, 60.4±5.3 and 60.0±5.1%, respectively ($n = 10$) within 48 h. It also inhibited the late long-term Na^+,K^+ pump activation, as determined from the ouabain-sensitive Rb uptake, and prevented the increase in the intracellular K content at the late stages of $G_0/G_1/S$ progression. Cyclosporin A did not affect the early transient pump activation, the dynamics of ouabain-resistant influxes and the intracellular Na content in PHA-activated lymphocytes. When added 1 h after PHA, CsA neither affected the activation of the pump-mediated Rb influxes nor the increase in the intracellular K content. It is concluded that in activated human lymphocytes, the long-term activation of Na^+,K^+ pump associated with the mitogen-induced blast transformation, as well as the late increase in K content depend on the T-cell growth factor interleukin-2.

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Blast transformation of human peripheral blood lymphocytes, induced by phytohemagglutinin (PHA), is accompanied by a long-term activation of Na^+,K^+ pump which occurs at the stage of lymphocyte growth and characterizes the transition of quiescent cells to proliferation [1]. In cultured cells of established cell lines, a stable increase of potassium fluxes through the pump was also found to be related to the change in the level of cell proliferation induced by various mitogens [2–5]. A short-term increase of

ouabain-sensitive K^+ influxes at the initial stage of the response to the mitogenic stimulus, shown earlier for various cell types by many investigators, and the long-term increase of K^+ fluxes via Na^+,K^+ pump in transition from quiescence to proliferation differ in a number of features [6–10]. In particular, it has been shown that a long-term increase of ionic pump activity which accompanies the blast transformation of activated human lymphocytes, in contrast with the fast rise of the fluxes at the early stage of activation, is not related to the increase of the intracellular Na^+ concentration, depends on protein synthesis and is suppressed by transcription inhibitors [11, 12]. The data obtained in studies of the dynamics of ionic fluxes in activated lymphocytes, suggest that an increase in the transport activity of Na^+,K^+ pump at later stages of the $G_0/G_1/S$ progression should be provided by new ionic pumps, the synthesis of which can be controlled at the genome level.

Cyclosporin A (CsA), a known immunosuppressing drug, inhibits the transition of quiescent T cells to proliferation by preventing the production of lymphokines, in particular, by inhibiting the production of T-cell growth factor interleukin-2 (IL-2) [13–15]. Inhibition of IL-2 expression by cyclosporin A at the early stage of the proliferative response of lymphocytes is due to the selective blockage of the transcription factor NF-AT resulting from the binding of CsA to K^+-dependent phosphatase – calcineurin [16–18]. CsA (as another immunosuppressant, FK-506) attracts the attention of many researchers as a tool which makes it possible to determine the potassium-dependent pathway of activation of lymphoid cells and to switch off the IL-2-dependent response of T cells to mitogens. In this work, we used CsA to elucidate the role of early activation events in triggering the lymphocyte response to the mitogen stimulus, in particular, expression of IL-2, in the late activation of Na^+,K^+ pump. The results of the study show that in PHA-stimulated human lymphocytes CsA inhibits that long-term increase of the functional activity of the ionic pump, which accompanies the transition of the quiescent cells to proliferation.

EXPERIMENTAL

We used the following reagents: ouabain (Sigma, USA); phytohemagglutinin (Calbiochem, USA); dextran 500 (Pharmacia, Sweden); ficoll 400, medium RPMI (Flow, Scotland); verografin, 76% (Spofa, Czech Republic); isotopes (Izotop, Russia); cyclosporin A (Sandoz, Switzerland). Other reagents used were of chemical purity or special purity grade (made in Russia). Cyclosporin A was dissolved in 96% ethanol and diluted to a final concentration of 1 μg/ml immediately before the experiment. The final concentration of ethanol in samples did not exceed 0.05%.

Isolation and cultivation of lymphocytes. The experiments were carried out on human peripheral blood lymphocytes. The lymphocytes were isolated according to the conventional protocol [19] from the fresh donor blood using Glygicir as an anticoagulant. The erythrocytes were precipitated by adding a 6% dextran solution into the blood; the leucocyte fraction was taken in 10–15 min. The lymphocyte suspension (3 ml per each sample) was put on top of an 8-ml ficoll–verografin medium (density, 1.077) and centrifuged at 400 *g* for 30 min. After centrifugation, the interphase was transferred into 12 or 50 ml test tubes and washed three times with the physiological saline of the following composition (mM): NaCl, 137; Na_2HPO_4, 8; KCl, 2.7; KH_2PO_4, 1.5. The lymphocytes were put into 50–100-ml flasks filled with 20–30 ml of the medium RPMI-1640 with glutamine and 10% human serum ABIV, Rh(+) without antibiotics at a concentration of 1.5–2 million cells per 1 ml. After an incubation, the lymphocyte suspension was dispensed into 10-ml vials at a concentration of 2–4 million cells in 2 ml of the same incubation medium. PHA was then added at 20 μg/ml.

Measurements of Rb influxes, intracellular K and Na contents and rates of protein, RNA and DNA syntheses. To measure the fluxes and intracellular contents of cations, and also the proliferation parameters in PHA-stimulated lymphocytes, vials with cell suspension were taken at certain time intervals and appropriate labels were added. In a typical experiment, measurements were performed 0.5, 2, 5, 8, 16, 24, 48 and 72 h after PHA had been added to the culture. The K^+ influx was assessed by the accumulation of Rb which is a physiological analogue of potassium for the ion transport systems studied [20]. To measure the influx, RbCl at a final concentration of 2.5 mM was added into some vials (n = 5–7); the lymphocyte suspension was incubated for 30 min in a CO_2 incubator at 37°C. To identify the flux due to Na^+,K^+ pump, ouabain at 0.1 mM was added into some vials with RbCl and the ouabain-sensitive flux was determined as the difference between the accumulation of rubidium in the absence and presence of ouabain. CsA at a concentration of 1 μm/ml was added to the lymphocyte culture 5–10 min before the application of PHA.

The rates of syntheses of protein, RNA and DNA were assessed using [^{14}C]leucine, [^{3}H]uridine and [^{3}H]thymidine, respectively. 0.1 ml [^{14}C]leucine (final concentration, 2.7 μCi/ml) and 0.1 ml [^{3}H]thymidine (final concentration, 10 μCi/ml) or 0.1 ml [^{3}H]uridine (final concentration, 10 μCi/ml) were added to the vials used for Rb flux measurements.

After a 30 min incubation with the labels, the lymphocyte suspension was transferred into 2 ml conic plastic test tubes, centrifuged for 30 min at 3000 *g*, the pellet was washed five times with a cold solution of $MgCl_2$ (85 mM) without resuspending the pellet, and then 1 ml of trichloroacetic acid (TCA) was added. The content of cations in the supernatant was determined using a Perkin-Elmer AA-306 atomic absorption spectrophotometer [21]. After the

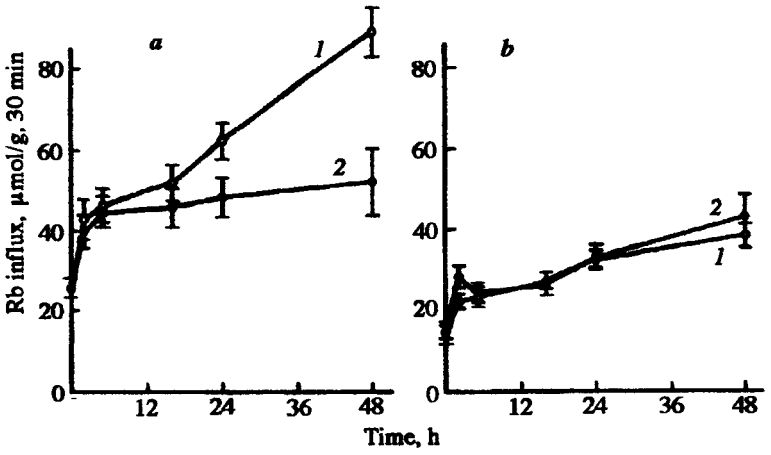

Figure 1. Effect of cyclosporin A (CsA) on the dynamics of ouabain-sensitive (*a*) and ouabain-resistant (*b*) Rb influx in human lymphocytes activated by phytoagglutinin (PHA). *1*, PHA; *2*, PHA + CsA. Mean ± SE (n = 10) are given.

measurements, the supernatant was discarded, the pellet was dissolved in 1 ml of NaOH (0.1 M) and used to measure the uptake of [^{3}H]thymidine by DNA, [^{14}C]leucine by protein and [^{3}H]uridine by RNA and also to measure the concentration of protein according to the Lowry method. The concentrations of cations – potassium, sodium and rubidium – in the lymphocytes, and also of the labels characterizing the rates of macromolecule synthesis, were calculated per 1 mg of cell protein for each sample. No less than five samples were taken for each time point. The quiescent lymphocytes were analyzed simultaneously with the first samples of the activated lymphocytes using the same experimental procedures.

Measurements of the size of lymphocytes. The quiescent and PHA-stimulated lymphocytes were classified by size using a Joyce-Loebl Magicscan 2 scanning microscope after they were fixed with a mixture of alcohol, formalin and acetic acid (9:3:1) and stained according to Gymza.

RESULTS

Rubidium influxes. Figure 1 shows the response of the ouabain-sensitive and ouabain-resistant lymphocytes to PHA (20 μg/ml) and cyclosporin A (1 μg/ml). CsA was added to the lymphocyte culture simultaneously with PHA.

In accordance with our earlier findings [1, 11], the fast phase of the Na^+,K^+ pump activation, which completes within the first two hours, is followed 16 h later by a prolonged increase of the ouabain-sensitive Rb influxes, which

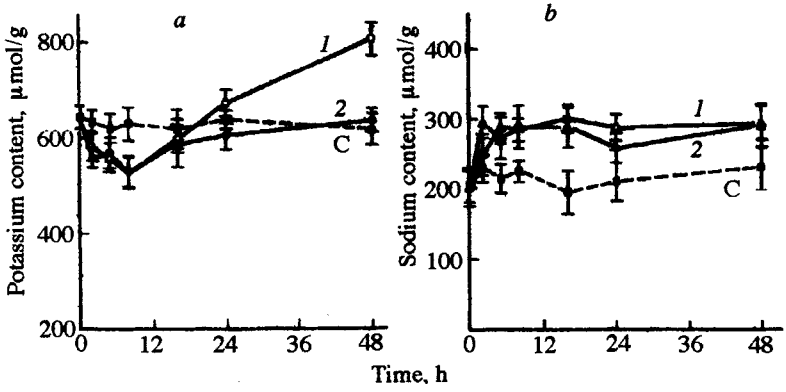

Figure 2. Effect of CsA on the intracellular K (*a*) and Na (*b*) contents in quiescent and PHA-activated lymphocytes: *1*, PHA; *2*, PHA + CsA; C, quiescent lymphocytes in medium RPMI with the serum but without PHA.

accompanies the stage of lymphocyte growth and covers the period preceding the DNA synthesis. As shown in Fig. 1*a*, CsA has no effect on the ouabain-sensitive Rb influxes during the first (fast) phase, but it prevents the increase of the fluxes during the second phase. 48 h after the addition of PHA, the ouabain-sensitive Rb influx increases about three times, whereas in the presence of CsA the flux does not virtually change between hours 16 and 48.

The dynamics of ouabain-resistant Rb influxes is not affected by CsA either at the early stage of PHA action, when the influx increased twofold, or at the late stage when it increased slowly and not so much as compared with the ouabain-sensitive influx (Fig. 1*b*).

Therefore, in activated lymphocytes the action of CsA on the Rb fluxes via Na^+,K^+ pump and on the ouabain-resistant fluxes is different. The major action of CsA consists in the complete inhibition of the increase of the ionic pump activity at the late stages of lymphocyte transition from quiescence to proliferation.

Intracellular K and Na contents. Triggering of proliferation in quiescent lymphocytes is accompanied with characteristic changes in the intracellular K^+ content. During the first 5–8 h of mitogenic stimulation the intracellular potassium decreases and subsequently begins to increase and, by the time of maximal proliferation (48–72 h), the K^+ content in the proliferating cells exceeds that in the quiescent lymphocytes by 30% [1]. It should be noted that the intracellular K content shown is calculated relative to the cell protein content, which means that the overall increase of K content per cell in the activated lymphocytes is much larger (3–4 times).

As shown in Fig. 2*a*, CsA added to a lymphocyte culture simultaneously with PHA has no effect on the dynamics of potassium content in the first

hours: in the presence of only PHA or PHA with CsA, by the 8th hour of activation the intracellular content of potassium decreased similarly – from 640 to 500 μmol/g protein. The significant difference in the K content in lymphocytes stimulated with PHA in the presence of CsA and without it is pronounced after 16 h. As seen in Fig. 2*a*, CsA prevents the increase of the K content in activated lymphocytes, which is characteristic of the second half of the prereplicative stage. We should note that in cultures of quiescent lymphocytes CsA had no influence on the intracellular K and Na contents for 36 h (Table 1). The cation contents (in terms of cell protein) in the non-activated lymphocytes cultured in a medium with CsA began to increase only by 48 h, but the total protein decreased by that time 1.5-fold as compared with the cultures without CsA.

The stimulation of quiescent lymphocytes by PHA is accompanied by an increase in the intracellular Na content during the first two hours (Fig. 2*b*). In the presence of CsA the initial increment of Na in the lymphocytes is larger. In an experiment illustrated by Fig. 2*b*, the Na content in the lymphocytes stimulated with PHA against the background of CsA was 16.5±1.8% greater than without CsA. At the same time, the presence of CsA in the culture had no effect on the content of Na in activated lymphocytes in the late prereplicative period, and it had no influence, either, on the level of intracellular Na in non-activated lymphocytes (see Table 1).

These data suggest that CsA suppresses the increase in intracellular K content characteristic of the activated human lymphocytes at the stage of blast transformation.

Table 1. Effect of cyclosporin A (CsA) on the long-term activation of ouabain-sensitive Rb influx and the increase of the intracellular K content at the stage of proliferation of competent human lymphocytes prestimulated by a small dose of PHA.

Conditions of experiment	Rb influx, μmol/g/30 min		Content of cations, μmol/g protein	
	ouabain-sensitive	ouabain-resistant	K^+	Na^+
Resting lymphocytes	22.3±1.2	17.4±2.0	600±35	245±12
Resting lymphocytes + CsA, 48 h	23.0±2.0	20.0±1.9	586±38	230±15
PHA, 20 μg/ml, 48 h	61.7±8.7	30.6±9.3	760±54	285±35
PHA, 20 μg/ml + CsA, 48 h	28.4±11.0	29.6±7.4	548±41	284±38
PHA, 2 μg/ml, 1 h, then PHA, 20 μg/ml + CsA, 47 h	54.2±9.1	26.5±3.5	690±43	278±29

Note: Data of a typical experiment (out of four experiments). The mean ± SE for three cultures of the lymphocytes of one donor are given.

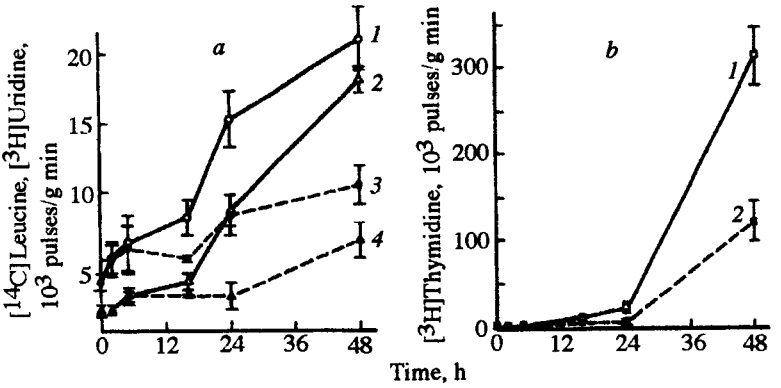

Figure 3. Effect of CsA on protein, RNA (*a*) and DNA (*b*) syntheses in PHA-activated human lymphocytes. *a*: *1*, *3*, [^{14}C]leucine; *2*, *4*, [^{3}H]uridine. The means and standard errors for 10 experiments are given; *1*, *2*, PHA; *3*, *4*, PHA + CsA; *b*: *1*, PHA; *2*, PHA + CsA.

Protein, RNA and DNA syntheses and lymphocyte growth. The effect of CsA on the dynamics of the syntheses of macromolecules in PHA-stimulated lymphocytes is shown in Fig. 3. CsA has no effect on the early increase in the rate of [^{14}C]leucine incorporation into protein for the first 8 h of the incubation with PHA, but it inhibits the increase in the uptake of [^{14}C]leucine 12 h after the application of PHA. As a result, the rate of protein synthesis in the presence of CsA is by 48 h twice as low as without CsA. In PHA-activated lymphocytes, the uptake of [^{3}H]uridine begins to increase already in the first hours, but the most significant increase is observed after 16 h. As it follows from Fig. 3*a*, CsA inhibits just the late enhancement of RNA synthesis in the activated lymphocytes. As for DNA synthesis, which was assessed by the rate of [^{3}H]thymidine uptake, by 48 h it was inhibited by 60.4±5.3% in the presence of CsA (Fig. 3*b*).

The growth dynamics of the lymphocytes after their stimulation with PHA is shown in Fig. 4. In the presence of CsA the growth of cell protein mass is inhibited by 48 h by half (Fig. 4*a*). As shown in Fig. 4*b*, in the presence of CsA the increase of the volumes of the activated lymphocytes is inhibited by 60.4±5.1% within the interval of 16–48 h. Thus, in PHA-activated human lymphocytes, CsA at a concentration of 1 μg/ml inhibits the growth-related increase of protein and RNA syntheses, suppresses DNA synthesis and prevents the increase of cell size.

CsA does not affect ionic changes in competent lymphocytes. According to the data reported earlier [22, 23], CsA prevents expression of IL-2 only when added simultaneously with or before PHA. However, CsA does not block the expression of the growth factor induced earlier and is not efficient at the stage

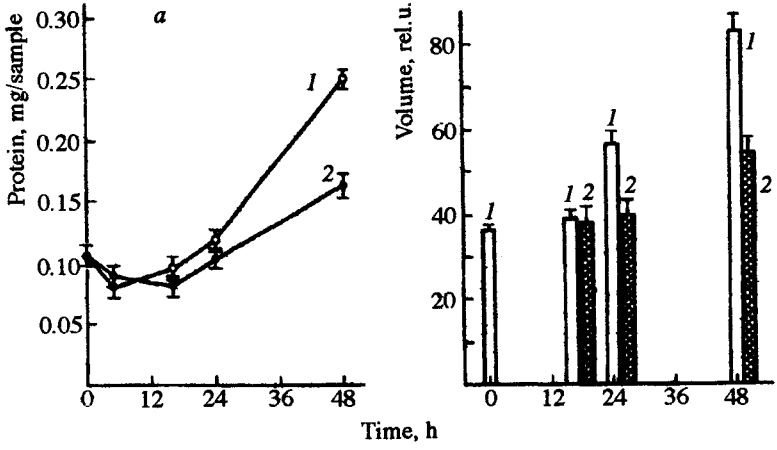

Figure 4. Effect of CsA on the increase of cell protein mass (*a*) and on the increase of the size of PHA-activated human lymphocytes (*b*). The sample contained $3 \cdot 10^6$ cells. The data of a typical experiment are given. *1*, PHA; *2*, PHA + CsA.

of the proliferation of the stimulated cells, either. In our experiments, isolated lymphocytes had been incubated for 1 h in a medium containing a subthreshold concentration of PHA (2 μg/ml) before PHA at a mitogenic concentration (20 μg/ml) was added together with CsA. Short-term applications of PHA induced expression of IL-2, i.e., made the cells "competent". However, to induce DNA replication and division of the cells thus activated, yet another stimulus is required, which can be PHA at a mitogenic concentration [22].

As shown in Table 1, CsA does not inhibit the rise of the ouabain-sensitive Rb influxes and the increase of the intracellular K content in the prereplicative phase in lymphocytes which became competent as a result of a treatment with a small dose of PHA. We should note that in the experiments carried out using this protocol, the rates of protein and RNA syntheses on the second day of mitogenic activation of lymphocytes increased slightly slower than in control cultures stimulated only with PHA. Thus, in an experiment presented in Table 1, in lymphocytes first treated with PHA at a small concentration and then stimulated by PHA in the presence of CsA, the uptake of [^{14}C]leucine by 48 h proved to be 10.2±1.9% lower, and [^{3}H]uridine, 17.2±2.4% lower than in lymphocytes stimulated only by PHA at a mitogenic concentration. At the same time, in cultures of lymphocytes successively treated with PHA and CsA, the proliferative response assessed by the uptake of [^{3}H]thymidine by 48 h of stimulation was high and did not differ from that of the lymphocytes activated by PHA without CsA. Unexpectedly, under the conditions of the lymphocyte activation used in each

of the four experiments, the increase of the mass of cell protein and of the lymphocyte size was lower by 30–40%. What caused the observed difference of the growth and proliferation parameters under these conditions is not clear. The results obtained in this series of experiments suggest that the long-term activation of the ionic pump, coupled with blast transformation, is somehow related with the initial stages of triggering the mitogenic response of the lymphocytes, when the dominating event is the expression of T-cell growth factor IL-2.

DISCUSSION

It is generally recognized that the mechanism of the cytostatic action of a known immunosuppressant cyclosporin A on lymphoid cells involves the blockage inhibition of the transcription factors, the regulation of expression of IL-2, due to the binding of CsA to the cytoplasmic protein, calcineurin [24]. Studies at a cell level showed CsA to be efficient only at the initial stage of the mitogenic response. It had no inhibitory effect if added to a lymphocyte culture later, at the stage of the proliferation of the activated cells [22, 25]. We used this ability of CsA to selectively switch off the transcription of IL-2 for studies of the assumed relation between the additional functional expression of the ionic pump at the later stages of $G_0/G_1/S$ progression of human lymphocytes stimulated by PHA, and the expression of the T-cell growth factor IL-2.

CsA was added to the lymphocyte culture simultaneously with or 1 h before PHA and the cells were incubated with CsA for 48 h. It can not be excluded that the basic parameters of ionic transport could be affected due to the prolonged presence of CsA rather than to the mitogenic activation. There are data in the literature on the side effects of CsA. For instance, CsA (in contrast with its inactive analogues) depolarizes the cell membrane, and this depolarization is not related to the antiproliferative effect of CsA [23, 26, 27]. As shown in our experiments, the presence of CsA in cultures of quiescent lymphocytes for 48 h has no effect on the values of ionic fluxes and the intracellular K and Na contents. Earlier works on the cytostatic action of CsA also showed it to be not toxic at concentrations of up to 1 μg/ml with respect to lymphoid cells [13, 28, 29].

The major result of the present work is that CsA at concentrations high enough to suppress the proliferative response of the lymphocytes to PHA eliminates the late activation of ionic pump. Also, the increase of intracellular K content characteristic of the development of the mitogenic response is absent or weakly pronounced in lymphocytes stimulated by PHA in the presence of CsA. At the same time, CsA does not affect other investigated parameters of ionic homeostasis – the intracellular content of sodium and ouabain-resistant transport. Such specific features in the action of CsA on the

dynamics of ionic parameters in activated lymphocytes indicates a relationship between the long-term activation of ionic pump, the intracellular K content and the proliferative response. The data obtained support the conclusions made in studies of ionic homeostasis in cultured cells of established cell lines that the high intracellular content of potassium and the high level of activity of the ionic pump are characteristic features of the proliferating cells [4]. As our earlier studies have shown, the increase in the content of potassium, observed in transition from quiescence to proliferation is not just the consequence of the increase of cell size, but is a specific increase of the intracellular content of potassium per 1 g of cell protein or, in fact, per 1 g of the dry weight of the cells [3].

In the presence of CsA, the inhibition of the syntheses of macromolecules in PHA-stimulated lymphocytes was, as a rule, incomplete. According to other reports [25, 30], the antiproliferative action of CsA depends on the type of mitogenic stimulus. In particular, the studies of the mechanisms controlling the expression of IL-2 mRNA showed that the IL-2 transcription induced by phorbol ester or phorbol ester in combination with antibodies αCD28 was not inhibited by such immunosuppressants as CsA or FK506. Production of IL-2 in this case is triggered via the CsA-resistant mechanism involving the activation of CD28 receptor [25, 30]. It was also shown that lectin-induced expression of IL-2 mRNA in lymphoid cells is switched off by CsA less efficiently than that induced by ionomycin or ionomycin in combination with phorbol ester [28, 30]. Presumably, the partial inhibition of the activation of lymphocytes observed in our experiments with CsA is due to the fact that just the CsA-resistant mechanisms provide the induction of IL-2 and trigger an incomplete mitogenic response.

The data of this study show for the first time that the sodium pump activation which accompanies the proliferation phase of the PHA-induced mitogenic response of the lymphocytes is inhibited by cyclosporin A. Taking into account the high specificity of CsA in the inhibition of expression of T cell growth factor, we suggest that the mechanisms responsible for the long-term activation of Na^+,K^+ pump during blast transformation are associated with the events which control the early triggering of IL-2. Indeed, recent studies have revealed the relations between the expression of IL-2 and the activation of some transport processes, functionally significant for the development of the complete response of the cells to the mitogenic signal. Thus, the activation of human lymphocytes is accompanied with the stimulation of glucose transport at the late stages of the transition from quiescence to proliferation, which is due to the change in the ratios of expression of various isoforms of glucose transporter [31, 32]. Expression of the isoform GLUT-1 characteristic of activated lymphocytes is inhibited by CsA and depends on the expression of IL-2 and its receptor, but the key event for the expression of GLUT-1 is, most probably, the interaction of IL-2 with

the receptor [32]. Our preliminary studies indicate that in PHA-activated human lymphocytes the level of mRNA of the $\alpha 1$ and $\beta 1$ subunits of Na^+,K^+-ATPase increases in the first 8 h [33]. We believe that the early expression of Na^+,K^+-ATPase in activated lymphocytes can explain the late increase of the K influxes, related to the blast transformation, as well as the rise of the activity of the ionic pump [12]. Further studies of the dynamics of expression of Na^+,K^+-ATPase, IL-2 and its receptor can reveal the concrete mechanism of the CsA-induced inhibition of the additional (related to the cell cycle) functional expression of ionic pump in activated lymphocytes.

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