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DNA Synthesis and Interferon Release by Human Peripheral Lymphocytes Exposed to High Potassium Medium[1] (42456)

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Abstract. Lymphocytes have their membrane potential changed during transition from the resting to the active state. On these grounds, we investigated the response of human peripheral lymphocytes to a depolarizing high-potassium (102.7 m*M*) medium, by assaying cellular incorporation of labeled thymidine and interferon (IFN) release in the culture medium. The greatest effect is evident 3–4 days after preincubation for 120 min in high K^+ medium. A temporal correlation has been observed between rate of DNA synthesis and IFN production. The IFN activity is shown to be γ-type. © 1987 Society for Experimental Biology and Medicine

Lymphocytes stimulated *in vitro* by a variety of agents undergo a complex series of metabolic processes (1–3) and changes of the membrane properties (4) that on the whole have been referred to as lymphocyte activation.

Although initially observed in the neuron, membrane potential changes have been stressed as mediating some events in lymphocyte activation. Epstein–Barr virus causes in receptor-bearing B lymphocytes but not in receptor-negative T lymphocytes a biphasic membrane potential change consisting in an initial hyperpolarization followed by a depolarization (5). On the contrary the lymphocyte mitogenic stimulation results sequentially in depolarization, repolarization, and hyperpolarization, and these changes are correlated with DNA synthesis (6, 7). Furthermore, lymphocyte cultures stimulated with T-cell mitogens release IFN-γ (8), a lymphokine with antitumoral and immunosuppressive activity greater than other IFNs (9).

On the basis of these findings, it seems likely that there is a relationship between transmembrane potential changes and subsequent biological responses.

The present study was undertaken to investigate the effect of a depolarizing medium (10–12) on the lymphocyte activation, revealed in the form of proliferative and antiviral activities.

Materials and Methods. *Lymphocyte preparation and culture.* Lymphocytes were separated by layering buffy coats of healthy donors (age range 30–40 years) over Ficoll–Hypaque (13). The technique routinely resulted in cell populations consisting of approximately 80% lymphocytes and 20% monocytes. Cell number was determined by light-microscopic count and viability was assayed by trypan-blue dye exclusion technique. The incubation was carried out in triplicate in sterile microtiter plates (12-well culture clusters; COSTAR) at 37°C in humidified atmosphere of 5% CO_2–95% air; each well contained 1×10^6 pooled cells/ml in RPMI 1640 medium (GIBCO), pH 7.2–7.4, supplemented with 20 m*M* glutamine (GIBCO), 10 m*M* Hepes (GIBCO), 1% penicillin–streptomycin solution (Labtek, Eurobio, France), and 10% heat-inactivated fetal calf serum (GIBCO). The composition of normal, unsubstituted RPMI 1640 medium was 102.7 m*M* NaCl, 5.4 m*M* KCl, 0.4 m*M* $MgSO_4$, 0.4 m*M* Ca $(NO_3)_2$ $7H_2O$, 5.6 m*M* Na_2HPO_4, and 11 m*M* glucose.

Activation of lymphocytes. Lymphocytes were cultured for 30, 60, 90, and 120 min in RPMI 1640 Select-Amine Kit medium (GIBCO) prepared with 102.7 m*M* KCl replacing 102.7 m*M* NaCl (high-K^+ medium). At the end of each time period the cultures were collected and centrifuged at 400*g* for 10 min at 20°C. The pellet was resuspended in

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RPMI 1640 and incubated at 37°C for 96 hr. Control wells in each plate contained unstimulated cells and cells stimulated with staphylococcal enterotoxin A (SEA; SIGMA) added at a dose of 0.1 μg/10^6 cells at the start of the culture. IFN activity was assayed in supernatants and [^{3}H]thymidine incorporation in pellets after 24, 48, 72, and 96 hr.

Measurement of DNA synthesis. DNA synthesis was assayed by use of [^{3}H]thymidine according to Peavy *et al.* (14). A dose of 1 μCi of [*methyl*-^{3}H]thymidine (sp act 1 Ci/mmol; Sorin Biomedica, Italy) was added to each well 24 hr before harvesting. Incorporation then was determined by liquid scintillation counting. Data are expressed as the difference in counts per minute (cpm) between the means of stimulated and unstimulated cultures.

Antiviral assay. Supernatants were titrated for IFN activity with a microplaque reduction assay (15) using human epidermoid carcinoma larynx cells (HEp 2) and human amnionic cells (Wish), and vesicular stomatitis virus (VSV, Indiana strain) as challenge virus. Cell monolayers were infected with 50 plaque-forming units of VSV and all samples were tested at least twice in quadruplicate. Titrations always were made employing the international reference preparation (IRP) for human IFN-γ (Gg 23-901-530, NIAID, NIH, Bethedsa, MD).

Characterization of the antiviral activity. The characterization of antiviral activity produced by lymphocytes included acidification of samples at pH 2.0 with 0.1 *M* HCl, followed by neutralization at pH 7.0 prior to the assay, heating at 56°C for 1 hr, dialysis for 1 day at 2°C, ultracentrifugation at 105,000*g*, and proteolytic treatment (trypsin: 0.25% for 30 min at 37°C).

Neutralization of IFN was carried out as follows. Concentrated samples (50 μl, 150 units/ml) as well as IFN international standards were incubated (60 min at 37°C) in the presence of individual and pooled anti-IFN antisera and the residual antiviral activity was then assayed. Anti-IFN-α and -IFN-β antisera (G-026-502-568 and G-028-501-568, obtained from NIAID, NIH) were used at a dilution of 1:2000 and 1:40, respectively. Anti-IFN-γ antiserum (kindly provided by Professor J. Vilček and Dr. M. P. Langford, with a neutralizing titer of about 1:2500 and 1:10,000 against 10 units/ml, respectively) was used at a dilution 1:20 (16). For each experiment the amounts of anti-human IFN-α, -β, and -γ used were sufficient to completely neutralize the corresponding IFNs.

Results. *Cell responsiveness in control cultures.* The ability of the culture system to support lymphocyte transformation and lymphokine production was established by means of the specific T-lymphocyte mitogen SEA (17) added at the start of the culture. The results in Fig. 1 show both a continuous IFN release and [^{3}H]thymidine incorporation. In addition, the maximum IFN level was detected at 72–96 hr of culture and coincided with the greatest proliferative response.

On the other hand, unstimulated cells produced only negligible amounts of IFN (10 IU/

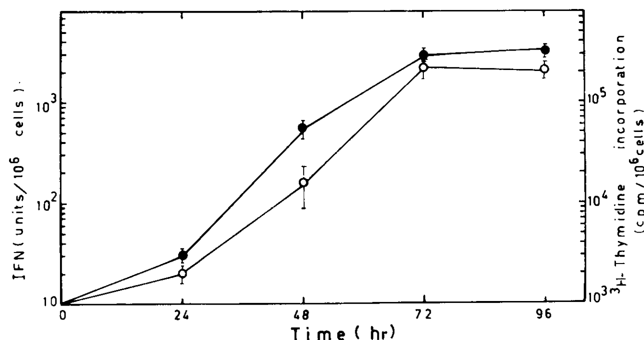

FIG. 1. Effect of SEA on lymphocyte activation. Cells were cultured (37°C, 5% CO_2 incubator) at 1 × 10^6 cells/ml in RPMI 1640 plus 10% FCS, pH 7.2–7.4, in presence of 0.1 μg/10^6 cells SEA. At the times indicated the rate of [^{3}H]thymidine uptake (●) and IFN production (○) were measured as described under Materials and Methods. Values are means ± SD for triplicate samples for five experiments.

EXPOSURE (minutes) TO HIGH-K⁺MEDIUM | IFN PRODUCTION (units/10⁶ cells) | ³H-THYMIDINE INCORPORATION (cpm/10⁶ cells)

200 400 600 800 1000

10^5 2.10^5 3.10^5

30

60

90

120

FIG. 2. Effect of high-K^+ medium on lymphocyte activation. Cells were preincubated (37°C, 5% CO_2 incubator) at 1×10^6 cells/ml in high-K^+ medium for 30, 60, 90, and 120 min. At each time the cells were collected and resuspended in RPMI 1640 plus 10% FCS, pH 7.2–7.4. After 72 hr incubation the rates of [^{3}H]thymidine uptake and IFN production were measured as described under Materials and Methods. Values are means ± SD for triplicate samples for five experiments.

10^6 cells) and responded poorly (3×10^3 cpm/10^6 cells) in proliferation assay.

Effect of high extracellular potassium on cultured lymphocytes. Human peripheral lymphocytes preincubated in high-K^+ medium (102.7 m*M* KCl) for 30, 60, 90, and 120 min and cultured without further stimulation in RPMI 1640 for a suitable time (11, 18, 19) take up labeled thymidine and release IFN.

As shown in Fig. 2, the time course of lymphocyte reponsiveness is related to the length of preincubation time and a steady-state level is reached when the cells are exposed for at least 90 min to the medium containing the high K^+ concentration. Furthermore, there is a temporal correlation between IFN production and the rate of DNA synthesis. However the lack of a time lag between DNA synthesis and IFN-γ production may suggest an increased transcription and translation of existing DNA.

In order to determine the kinetics of the response, lymphocytes maintained for 120 min in high-K^+ medium subsequently were cultured without further stimulation in RPMI 1640 over a period of 4 days.

The data presented in Fig. 3 indicate that IFN production and thymidine incorporation exhibit a similar slope and that the lymphocyte response reaches peak level after 72 hr of in-

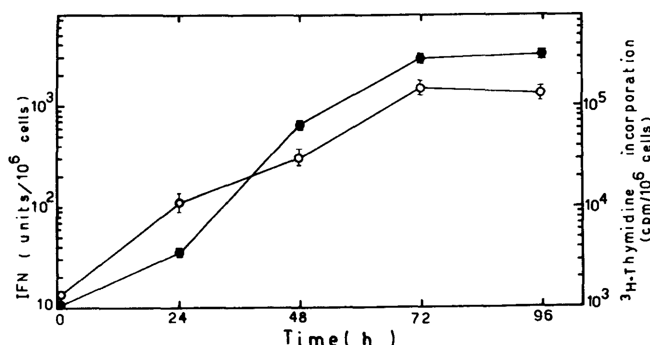

FIG. 3. Time course of [^{3}H]thymidine uptake and IFN production by cultured human peripheral lymphocytes. After 120 min preincubation in high-K^+ medium the cells were collected, resuspended in RPMI 1640 plus 10% FCS, pH 7.2–7.4, and incubated (37°C, 5% CO_2 incubator) for 24, 48, 72, and 96 hr. At each time the [^{3}H]thymidine incorporation (●) and IFN production (O) were measured as described under Materials and Methods. Values are means ± SD for triplicate samples for five experiments.

cubation. Negligible IFN amounts and low levels of incorporation were found in control unstimulated samples.

The findings that antiviral activity produced in our conditions was entirely acid labile and was neutralized only by anti-human IFN-γ serum suggest that it can be attributed to IFN-γ.

Discussion. Evidence has been accumulated recently suggesting that mitogenic stimulation of lymphocytes is associated with changes in ion fluxes across the membrane and consequentially with membrane potential variations which may represent an important early step in cell activation (6). Furthermore a clear indication exists that DNA and protein synthesis are strongly dependent on extracellular concentrations of potassium and sodium ions. In fact isotonic replacement of K for Na prevents low-Na inhibition of DNA synthesis (11). Additionally culture in high-K^+ medium modifies intracellular ion concentration (20) and ratios (21) and increases the mobility of membrane proteins, and these events could be required to trigger activation (22, 23).

On account of these findings we investigated whether a depolarization by means of cell exposure to a high-K^+ medium (11, 12) could mimic the biological action of mitogens.

The results show that the high extracellular potassium promotes cellular activation as pointed out by the correlation among the length of preincubation in high-K^+ medium, DNA synthesis, and IFN production. Treatment for 90–120 min is probably needed for a full depolarization to occur and it is noteworthy, with regard to the time course of depolarization, that it can vary after addition of mitogens up to 2–4 hr (6).

Additionally, the time course of [^{3}H]thymidine uptake and IFN release demonstrates that a substantial response can be obtained only after 3 to 4 days of culture in RPMI 1640, following preincubation in high-K^+ medium. Consistent with the events of the mitogenic stimulation of lymphocytes (6) we would predict that this time may be required for the full development of the synthetic capacity which reached its maximum after 72 hr.

The amounts of IFN released by lymphocytes in our experimental conditions are approximately the same as those obtained with mitogens (19, 24). Although there is no direct evidence, the results suggest a similar pathway when lymphocytes are exposed either to mitogens or to high extracellular potassium. On the other hand we do not know whether the effects are specific for K^+ or whether other ions can mimic *in vitro* its action.

1. Ling NR, Kay JE. Lymphocyte stimulation. North-Holland, Amsterdam, 1975.
2. Wedner HJ, Parker CW. Lymphocyte activation. Prog Allergy **20**:195–300, 1976.
3. Deutsch C, Taylor JS, Price M. pH homeostasis in human lymphocytes: modulation by ions and mitogen. J Cell Biol **98**:885–894, 1984.
4. Tsien RY, Pozzan T, Renk TJ. T-cell mitogens cause early changes in cytoplasmic free Ca^{2+} and membrane potential in lymphocytes. Nature (London) **295**:68–71, 1982.
5. Rosenthal KS, Shapiro HM. Cell membrane potential changes follow Epstein-Barr virus binding. J Cell Physiol **117**:39–42, 1983.
6. Kiefer H, Blume AJ, Kaback HR. Membrane potential changes during mitogenic stimulation of mouse spleen lymphocytes. Proc Natl Acad Sci USA **77**:2200–2204, 1980.
7. Kiefer H, Schulze R. The membrane potential of lymphocytes changes only in response to specific stimulation. Biosci Rep **2**:583–588, 1982.
8. Epstein LB. The comparative biology of immune and classical interferons. In: Cohen S, Pick E, Oppenheim J, Eds. Biology of the Lymphokines. New York, Academic Press p443, 1979.
9. Baron S, Dianzani F, Stanton GJ. General considerations of the interferon system. In: Baron S, Dianzani F, Stanton GJ, Eds. The Interferon System. Galveston, University of Texas Medical Branch, Part 1, Vol 41: p1, 1982.
10. Deutsch C, Holian A, Holian SK, Daniele RP, Wilson DF. Transmembrane electrical and pH gradients across human erythrocytes and human peripheral lymphocytes. J Cell Physiol **99**:79–94, 1979.
11. Deutsch C, Price M. Role of extracellular Na and K in lymphocyte activation. J Cell Physiol **113**:73–79, 1982.
12. Sinback CN Jr, Coon HG. Electrophysiological and pharmacological properties of cultured rat thyroid cells. J Cell Physiol **112**:391–402, 1982.
13. Boyum A. Isolation of mononuclear cells and granulocytes from human blood. Scand J Clin Invest **21**: 77–89, 1968.
14. Peavy DL, Adler WH, Smith RT. The mitogenic effects of endotoxin B on mouse spleen cells and human peripheral lymphocytes. J Immunol **105**:1453–1458, 1970.
15. Langford MP, Weigent DA, Stanton GJ, Baron S.

Virus plaque-reduction assay for interferon: Microplaque and regular macroplaque reduction assays. In: Pestka S, Ed. Methods in Enzymology. New York, Academic Press, Part A, Vol 78:p339, 1981.
16. Langford MP, Weigent DA, Georgiades JA, Johnson HM, Stanton GJ. Antibody to staphylococcal enterotoxin A-induced human immune interferon (IFNγ). J Immunol **126**:1620–1621, 1981.
17. Langford MP, Stanton GJ, Johnson HM. Biological effects of staphylococcal enterotoxin A on human peripheral lymphocytes. Infect Immun **22**:62–68, 1978.
18. Farrar WL, Johnson HM, Farrar JJ. Regulation of the production of immune interferon and cytotoxic T lymphocytes by interleukin-2. J Immunol **126**: 1120–1125, 1981.
19. Svedersky LP, Hui A, May L, McKay P, Stebbing N. Induction and augmentation of mitogen-induced immune interferon production in human peripheral blood lymphocytes by N^{α}-desacetylthymosin α_1. In: Khan A, Hill NO, Eds. Human Lymphokines. New York, Academic Press, p125, 1982.
20. Lubin M. Intracellular K and macromolecular synthesis in mammalian cells. Nature (London) **213**:451–453, 1967.
21. Shinohara T, Ptiatigorsky J. Regulation of protein synthesis, intracellular electrolytes and cataract formation in vitro. Nature (London) **270**:406–411, 1977.
22. Eididin M, Wet T. Diffusion rates of cell surface antigens of mouse-human heterokaryons. J Cell Biol **75**: 483–489, 1977.
23. Pool TB, Cameron IL, Smith NKR, Sparks RL. Intracellular sodium and growth control. In: Cameron IL, Ed. The Transformed Cell. New York, Academic Press, p397, 1981.
24. Diezel W, Rentz E, Ermolin G, Sakandelidze OG, Sonnichsen N. Augmentation of mitogen-induced interferon production in lymphocytes by thymosin. Exp Clin Endocrinol **84**:99–104, 1984.

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