Kinetics of the Zn^{2+}-Stimulation of Human Peripheral Lymphocytes *in Vitro* (35733)

HARTMUT RÜHL, HOLGER KIRCHNER, AND GUDRUN BOCHERT
(Introduced by S. E. Mergenhagen)

Department of Hematology, Free University of Berlin, Klinikum Steglitz, Berlin, Germany

Zinc compounds have been shown to induce mitoses in cultures of normal human peripheral lymphocytes (1). Such stimulated lymphocyte culture represents an ideal model to investigate control factors of DNA synthesis and mitosis. The investigation of most of the already described stimulants (2) is complicated by their macromolecular structure. Therefore further experiments concerned with the kinetics of the lymphocyte response to zinc were undertaken.

Material and Methods. Our lymphocyte culture system has been described (3). In these experiments Eagles minimal essential medium (Flow Lab., plus 2 m*M* glutamine, without antibiotic) and 30% autologous plasma were used; and the cells were cultivated in 16 × 125-mm disposable plastic tubes (Falcon) in an atmosphere of 5% CO_2 in air for 144 hr. Quadruplicate 3-ml cultures received 2 μCi of ^{3}H-thymidine (TdR^3H, sp act 2 Ci/mmole) for the final 4 hr of incubation and were then put in an ice bath. After two washes with 0.9% NaCl the acid-insoluble material was precipitated twice with 2 ml of cold 5% trichloroacetic acid and once with 2 ml of cold absolute ethanol. The final precipitate was dissolved in 0.5 ml of Hyamine (Packard) plus 15 ml of standard scintillation solvent and counted in a Tricarb spectrometer model 3380 (Packard). Results (mean counts/min/culture = cpm) were only accepted when quadruplicate cultures were within ± 10% of their mean. For preparing autoradiographs, duplicate 3-ml cultures received 2 μCi of TdR^3H for the final hour; and smears were prepared and fixed 15 min with absolute methanol. They were further processed with K 5 emulsion (Ilford) according to a standard technique (4) and 1000 cells/smear were counted to determine the percentage of labeled nuclei. Mitotic index (0.1 y colchicine/ml/4 hr) and percentage of blast cells were evaluated in May–Gruenwald–Giemsa stained smears according to previously described criteria (3). Dye exclusion tests were made with trypan blue (30 min, × 37°, 0.1% solution). Zinc compounds were dissolved in a mixture of 9.5 ml of 0.9% NaCl and 0.5 ml of 0.1 *N* HCl, sterilized by filtration and added at the beginning of the cultures. Healthy adult blood or human cord blood was used for these studies.

Results. In this series of experiments, all $ZnCl_2$-treated lymphocyte cultures showed an increased uptake of TdR^3H on the sixth day

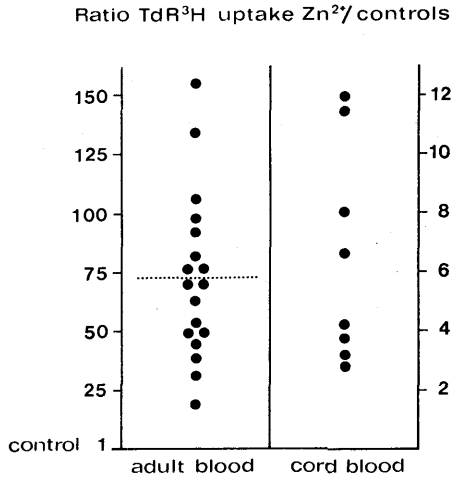

FIG. 1. Stimulation of human peripheral lymphocyte cultures (adult and cord blood) by $ZnCl_2$. Concentrations between 1.75 and 3.5 × 10^{-4} *M* were tested in each experiment and every point represents the ratio between optimal response to control of a donor, as measured by the total uptake of TdR^3H on the sixth day.

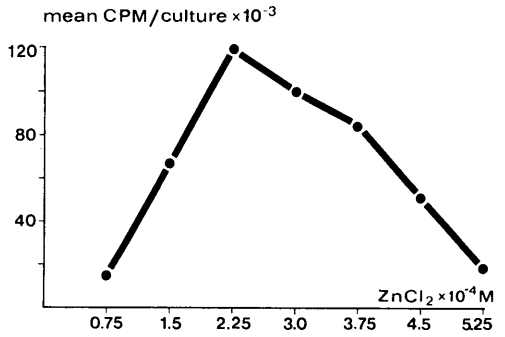

FIG. 2. Effect of zinc chloride on human peripheral lymphocyte cultures as measured by the total uptake of TdR^3H during the final 4 hr of the sixth day (untreated controls: 1634 cpm).

when compared with untreated controls. The ratio of increase ranged from 21 to 155 (mean 72) in the 18 adult individuals tested. In cultures of human cord blood TdR^3H uptake of untreated control cultures was between 3 and 10 times as high as in adult blood. Therefore, even though the absolute values of zinc-stimulated cord blood cultures were comparable with those of adult blood, the ratio of increase ranged from 3 to 12 (Fig. 1). To compare the action of zinc acetate, zinc aspartate, zinc chloride, and zinc sulfate, the blood of only one donor was used to determine the dose–response curve of all four compounds. Total uptake of TdR^3H was measured in quadruplicate cultures on the sixth day. The dose–response curve of zinc chloride in a typical experiment is given in Fig. 2 and we found that all four compounds produced optimal stimulation at the same concentration ($2.25–2.75 \times 10^{-4}$ *M*) in cultures of this donor (Table I). In another group of experiments cultures were set up with 2.25×10^{-4} *M* zinc chloride and were harvested daily from the second to the eighth day. It was shown in these experiments by the trypan blue method that the percentage "dead" cells in $ZnCl_2$-treated cultures was never higher than in untreated controls. There were few blast cells and little DNA synthesis up to the third day; the highest proportion of blast cells, mitoses, and TdR^3H-labeled cells and the maximal uptake of TdR^3H were found on the sixth day (Fig. 3a–d).

TABLE I. Optimal Concentration of Four Zinc Compounds to Induce DNA Synthesis in Human Peripheral Lymphocyte Cultures When the Blood of Only One Donor Was Used for Four Dose–Response Curves.

Total uptake of TdR^3H was measured during the final 4 hr on the sixth day.

	Conc[a] ($\times 10^{-4}M$)	Response[a, b]
Zinc acetate	2.75	101,245
aspartate	2.5	117,155
chloride	2.25	120,480
sulfate	2.5	102,280
No additive	—	1634

[a] Maximum of a dose–response curve.

[b] Mean of quadruplicate cultures (cpm/culture).

Finally, $ZnCl_2$-treated (2.5×10^{-4} *M*) lymphocyte cultures were incubated for different periods and then washed twice with warm culture medium to remove the stimulant. They were further cultured in medium with 30% autologous plasma, to which no zinc was added, and harvested on day 6. Results of this experiment (Table II) show, that the response of the cultures to zinc on the sixth day is greatly diminished, when they are washed between 24 and 72 hr. When the stimulant is removed after an interval of 96 hr, response is only 40% of untreated zinc cultures. Controls, which were washed in the same manner, but received fresh medium *with* $2.5 \times 10^{-4}M$ Zinc chloride, only showed slightly reduced TdR^3H-uptake on the sixth day.

Discussion. By measuring the total uptake of TdR^3H in the acid-insoluble fraction, it was shown that there was increased DNA synthesis in all investigated lymphocyte cultures of normal individuals when treated with $ZnCl_2$. Other zinc compounds had the same effect at the same concentration (Table I) and it therefore can be concluded that this is an effect of the zinc ion. Since blastic transformation and mitoses were found in Zn^{2+}-treated cultures of all healthy adults in this study and cord blood cultures were stimulated as well (Fig. 1), the mechanism of zinc stimulation is apparently a nonspecific one (5). The kinetics of the response, however, are more akin to those of antigen-stimulated

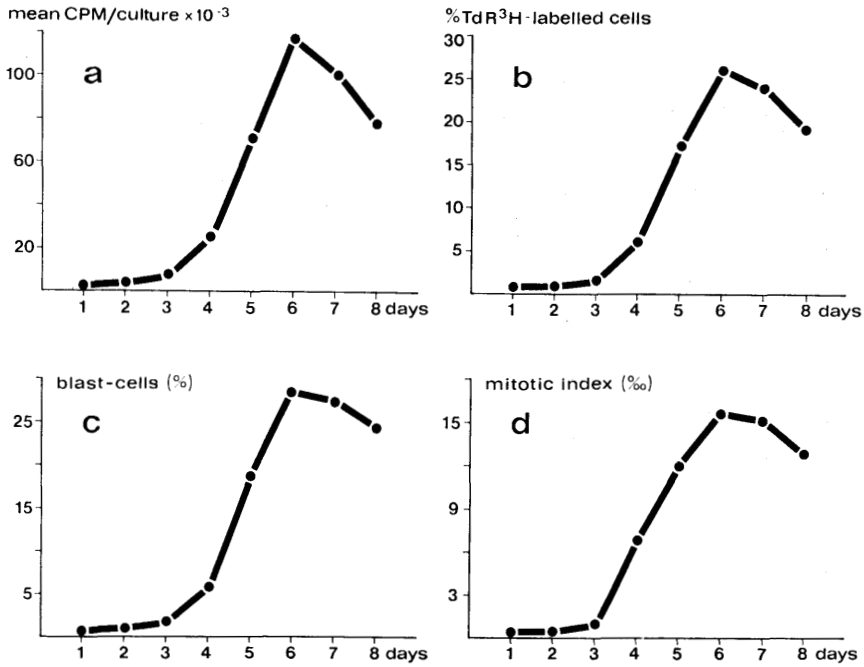

FIG. 3. Effect of 2.25×10^{-4} *M* zinc chloride on human peripheral lymphocyte cultures as measured by the total uptake of TdR^3H (a); percentage TdR^3H-labeled cells (b); percentage blast cells (c); and mitotic index (d). Control values were: 980–2170 mean cpm (a); 0.02–0.1% (b); 0.05–0.1% (c); and 0.0‰ (d).

lymphocyte cultures (up to 30% blast cells within 5 to 7 days).

Such kinetics seem to be typical for a weak stimulant whether specific or nonspecific in type, that stimulates only a few blast cells by the third day. From the experiments where the stimulant was removed by washing after different intervals (Table II), it can be concluded that Zn^{2+} must be in contact with the cells for a long period to obtain maximal stimulation on the sixth day.

TABLE II. Lymphocyte Cultures, Stimulated with 2.5×10^{-4}*M* Zinc Chloride, Were Washed at Different Times and Received Fresh Medium with (A), and without (B), 2.5×10^{-4}*M* $ZnCl_2$.

Time (hr)	A (mean cup)[a]	B (mean cup)[a]
24	112,737	1996
48	118,610	2340
72	115,336	12,237
96	107,480	46,732
Not washed, no additive[a]	648	
2.5×10^{-4}*M* $ZnCl_2$[a]	120,480	

[a] TdR^3H-uptake was measured in quadruplicate cultures after 144 hr.

The kinetics of Hg^{2+}-stimulated lymphocyte cultures (6, 7) are similar to our results with Zn^{2+}. Hg^{2+} has been shown to be preferentially localized within the condensed heterochromatin part of the nucleus and it has been suggested that derepression of RNA synthesis on Hg-bound DNA templates might occur (8). A similar mechanism might explain lymphocyte stimulation by Zn^{2+}, which has been shown to react with DNA as well (9, 10). Other possibilities are that Zn^{2+} activates enzymes necessary for DNA synthesis (11) or that it acts indirectly by altering one of the serum proteins to which it is known to be bound (12).

Summary. Zn^{2+} induced DNA synthesis to a varying degree in all peripheral lymphocyte cultures from healthy adults and from human cord blood. The effect of zinc acetate, zinc aspartate, zinc chloride, and zinc sulfate was identical, optimal stimulant concentra-

tion ranging from 2.25 to 2.75 $\times$ 10^{-4} *M*. Zinc-treated cultures did not show more cytotoxicity than untreated controls. Zn^{2+} stimulated few blast cells and little DNA synthesis at 3 days, but mitotic response and DNA synthesis occurred on day 6. A long contact of the stimulant with the cells seems to be necessary to induce maximal DNA synthesis on day 6.

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