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Variations of DNA polymerases-α and -β during prolonged stimulation of human lymphocytes

(phytohemagglutinin/DNA replication/DNA repair)

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ABSTRACT **Stimulation of human lymphocytes with phytohemagglutinin is known to induce an increase in overall DNA polymerase activity (DNA nucleotidyltransferase; deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7). Previous work [Pedrali Noy, G., Dalprà, L., Pedrini, A. M., Ciarrocchi, G., Giulotto, E., Nuzzo, F. & Falaschi, A. (1974) *Nucleic Acids Res.* 1, 1183] has shown that two subsequent waves of induction of DNA polymerase can be observed in this system; a first wave occurs in parallel with the increase in DNA replication rate; a second one occurs when the DNA synthesis rate is returned to minimal levels; the second peak is parallel to a maximum in DNA ligase and DNase levels.**

In the present work we have measured the levels of the DNA polymerases-α and -β in phytohemagglutinin-stimulated lymphocytes during a 12-day period; both enzymes are present at detectable levels at time zero; in correspondence to the peak of DNA synthesis rate (between the fourth and fifth day) a peak of DNA polymerase-α is observed, increasing by a factor of approximately 20-fold over the zero time value; subsequently, the level of DNA polymerase-α decreases in parallel with DNA synthesis rate. The DNA polymerase-β is also increased in correspondence to the peak in DNA synthesis rate, but reaches its maximum at later times, between the eighth and tenth day of incubation.

The capacity of stimulated lymphocytes to perform repair synthesis following UV damage was measured in the same cells used for the enzyme activity determinations; this capacity also shows two maxima: a first one correlated with the peak in DNA replication rate, and a second one correlated with the peak of DNA polymerase-β.

These data suggest a certain tendency to the specialization of functions in human cell DNA polymerases; the α-enzyme seems mainly correlated with DNA replication, whereas the β-enzyme seems more correlated with the ability of the cell to perform repair type synthesis.

Human lymphocytes stimulated with phytohemagglutinin (PHA) represent a useful model system to obtain information on the role of the enzymes of DNA metabolism; the stimulated cells undergo dramatic variations in DNA replication rate (1), and any positively correlated variation of the level of a certain enzyme gives a strong inference for a function of that enzyme in DNA replication.

Several authors have shown that overall DNA polymerase (DNA nucleotidyltransferase; deoxynucleosidetriphosphate: DNA deoxynucleotidyltransferase, EC 2.7.7.7) levels increase markedly in parallel to DNA synthesis rate, and both parameters reach their maximum at the same time, between the second and fifth day of culture (2–6), in correspondence to the peak of mitoses. In human cells two main molecular species of DNA polymerase are known, defined as -α and -β (7–10); the α-enzyme is the most abundant one, has an $s^0_{20,w}$ of 6.5 S and is inhibited by *N*-ethylmaleimide; the β-enzyme has an $s^0_{20,w}$ of 3.4 S and is insensitive to *N*-ethylmaleimide. The molecular species of DNA polymerase which is mainly responsible for the increase observed in PHA-stimulated lymphocytes is the DNA polymerase-α (11), which is also the only one that increases in response to a variety of proliferative stimuli in different tissues (12). These observations give a strong indication in favor of a main role in DNA replication for the α-enzyme, whereas, the role of the β-polymerase is still obscure.

Previous work from this laboratory (13) has shown that prolonged stimulations of human lymphocytes with PHA elicited two subsequent waves of increase of overall DNA polymerase activity; the first one corresponded to the peak in DNA synthesis rate, whereas, the second wave occurred at a time when DNA synthesis rate had returned to very low values; also other enzymes of DNA metabolism (namely DNA ligase and two DNase activities) reached a maximum in synchrony with the second peak of overall DNA polymerase activity.

The meaning of the second wave of DNA enzymes is obscure, but its possible correlation with the physiological state of the cells could give information on the role of the enzymes. Conversely, an analysis of the molecular forms of DNA polymerase involved in the two processes may give indications on the main function of each type of molecule.

In this work we will present data showing that, in correspondence to the second wave of increase of enzymes, the β-polymerase is stimulated more than in the first wave, and may represent the prevalent polymerase form, in contrast to what happens at the time of maximum replication rate. The capacity of the lymphocytes to perform repair synthesis following UV irradiation is also at a maximum in correspondence to the maximum level of the β-polymerase.

MATERIALS AND METHODS

Cells. For each experiment the lymphocytes were separated from a human blood sample obtained from a normal donor; 13 independent cultures, containing 1×10^6 white cells per ml, were incubated for the times indicated in Figs. 1 and 3; PHA was added to all cultures (except the one corresponding to the zero time sample), as already described (13). For the determination of DNA synthesis rate 5×10^5 cells in 0.5 ml of culture were incubated for 3 hr with 5 μCi of [^{3}H]thymidine (20 Ci/mol, Radiochemical Centre, Amersham), collected on Millipore filters, and washed with phosphate-buffered saline, 5% trichloroacetic acid, and ethanol; the filters were then dried and the radioactivity was measured (13).

Abbreviations. PHA, phytohemagglutinin; CH, thymidine incorporation rate in presence of hydroxyurea in nonirradiated cells; UH, thymidine incorporation rate in presence of hydroxyurea in ultraviolet irradiated cells.

DNA Repair. The ability of the cells to perform repair synthesis was measured by the procedure reported by Stefanini *et al.* (14) in samples of about 5×10^5 cells in 0.5 ml of culture, irradiated with UV light (Philips TUV 15 W lamp; dose rate of 24 erg mm^{-2} sec^{-1}) (1 erg = 0.1 μJ) and subsequently incubated for 3 hr in the presence of 5 μCi of [^{3}H]thymidine and 1.2 mM hydroxyurea (Sigma Chem. Co.). Control samples were treated identically, except for irradiation. The repair activity was expressed as the difference between the values of [^{3}H]thymidine incorporation in presence of hydroxyurea in UV-irradiated (UH) and unirradiated control cells (CH).

Preparation of Cell Extracts and Enzyme Assays. Preparation of extracts and determination of DNA polymerases -α and -β were performed on the remaining cells essentially as described by Coleman *et al.* (16). To a typical sample of 0.050–0.100 ml of packed lymphocytes (between 1.5 and 6.0 $\times 10^7$ cells), 3 volumes of 0.25 M sucrose containing 50 mM Tris·HCl (pH 7.6), 25 mM KCl, and 5 mM $MgCl_2$ were added and the cells were broken by 35 strokes of a Teflon pestle in a glass homogenizer. To the homogenate potassium phosphate buffer (pH 7.2) was added to a final concentration of 0.25 M; the suspension was then centrifuged for 1 hr at 40,000 rpm in a Spinco rotor 50 at 0°. The supernatants were used for crude extracts assays; for the fractionation of the α- and β-polymerases 0.25 ml aliquots of the supernatant were dialyzed versus 0.5 M NaCl, 25 mM Tris·HCl (pH 8.0), 1 mM EDTA (pH 8.0), and 1 mM 2-mercaptoethanol (buffer A) and layered onto a 5 ml gradient between 5 and 20% sucrose in buffer A; after centrifugation at 0° for 20 hr at 40,000 rpm in a Spinco SW50 L rotor, 0.25 ml fractions were collected by peristaltic pump aspiration from the bottom of tubes. DNA polymerase activities were measured by the incorporation of [^{3}H]dTTP into acid-insoluble material using activated DNA as substrate. The concentration of deoxynucleoside triphosphates in the assay was 0.050 mM and [^{3}H]dTTP specific activity was between 500 and 1300 cpm/pmol. Aliquots of the reactions were spotted directly on GF/C fiber disks and processed for acid-insoluble radioactivity (16). One unit of enzyme activity is defined as 1 nmol of dTMP incorporated per hr. The β-polymerase was assayed in the presence of *N*-ethylmaleimide, which specifically inhibits the α-polymerase (9). The inhibition step was carried out by preincubation of the crude extracts and the gradient fractions at 0° for 30 min with 5 mM *N*-ethylmaleimide.

Terminal deoxynucleotidyl transferase was assayed according to Coleman *et al.* (16).

RESULTS

Variations of the two different molecular species of DNA polymerase during PHA stimulation.

Fig. 1 reports the results of an experiment in which the cells were analyzed for the variations of DNA synthesis rate, of DNA repair capacity, and of the levels of the two main DNA polymerases during 12 days of culture in presence of PHA. The DNA synthesis rate in this experiment reaches its maximum around the fifth day, and decreases later to levels lower than $1/10$ of the maximum. The ability of the cells to perform repair synthesis shows two main peaks, the first one approximately 1 day in advance of the peak in DNA replication rate, and a second, more pronounced one, 3 days later, when the overall DNA synthesis rate is in the decreasing phase. The variation with time in DNA repair capacity follows the same pattern with two different doses of radiation.

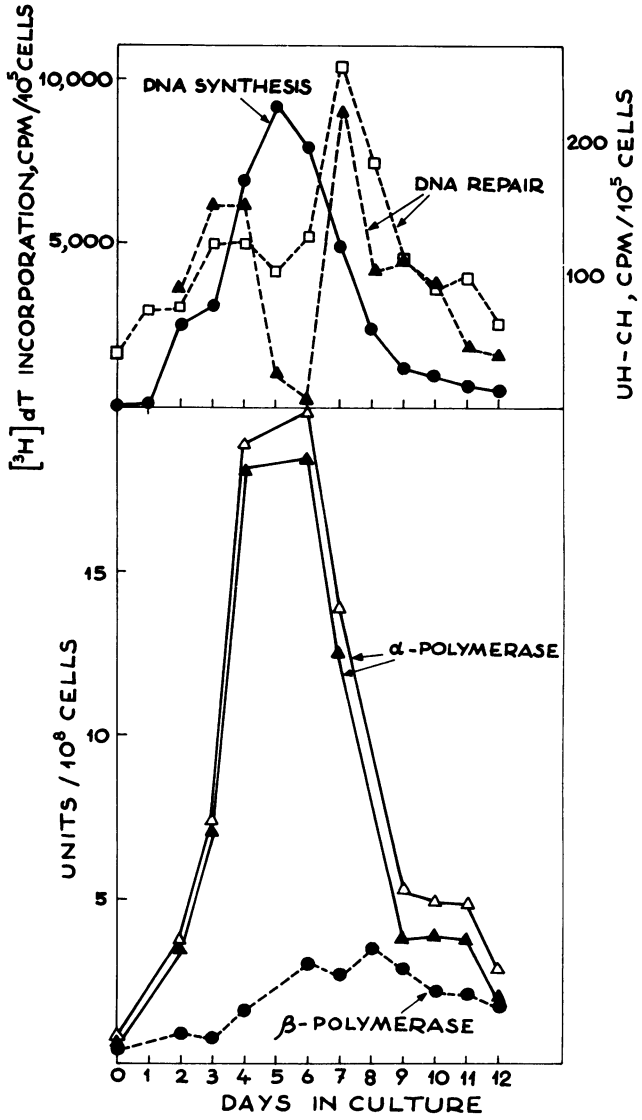

FIG. 1. Variations with time of DNA polymerase-α and -β activities, of DNA synthesis rate, and of DNA repair capacity in cultures of human lymphocytes stimulated with PHA. The procedures for the extraction and assays of polymerases are described in *Materials and Methods*. The values of α-polymerase assay are reported uncorrected and after correction for the contribution of the β-enzyme; DNA synthesis (●—●) was measured by thymidine pulses; DNA repair as the UV-induced thymidine incorporation in presence of hydroxyurea (UH) with respect to control (CH) (see *Materials and Methods*); □- - -□, data obtained with 720 erg/mm^2; ▲- - -▲, data obtained with 1080 erg/mm^2; △——△, α-polymerase uncorrected; ▲—▲, α-polymerase, after correction for the contribution of the β-enzyme; ●- - -●, β-polymerase.

The lower portion of the figure reports the results of the assays of α- and β-polymerases in the crude extracts. It must be pointed out that, whereas, the β-assay is strictly specific for this enzyme, the α-assay responds also, though to a reduced extent, to the β-enzyme. The data on the curve for the α-polymerase are accordingly presented also after correction for the contribution of the β-enzyme (which can be estimated as 50% of the activity measured in the conditions optimal for the latter enzyme). The α-enzyme is present at detectable levels at time zero, and rises in parallel with DNA replication rate, as reported by Coleman *et al.* (11), increasing by a factor of 24 over the zero time value; the maximum is reached between the fourth and sixth day, in synchrony

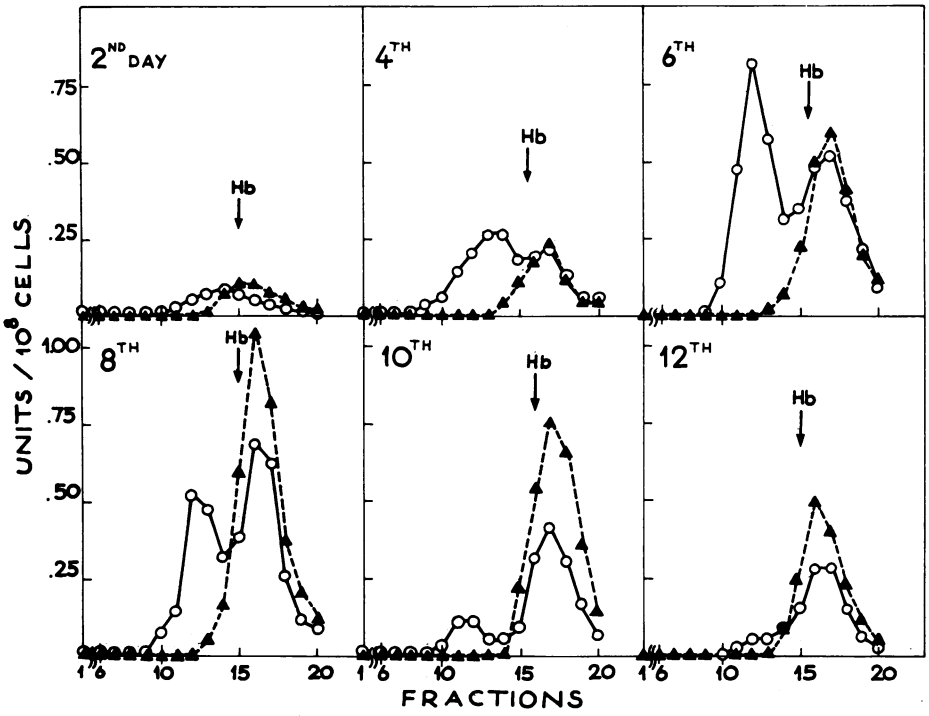

FIG. 2. Sucrose gradient fractionation of α- and β-polymerase activities at different times of stimulation of human lymphocytes with PHA. The fractionation was performed on the same extracts obtained for the experiments of Fig. 1. For the conditions see *Materials and Methods*. O—O, assay for α-polymerase; ▲- - -▲, assay for β-polymerase. The former assay reveals also the β-polymerase, though with lesser efficiency. The latter assay is absolutely specific for the β-enzyme. The sedimentation of marker hemoglobin in a control tube is indicated by arrows. Direction of sedimentation is from right to left.

with the DNA replication rate. The α-polymerase level decreases then sharply, returning to values of the order of $\frac{1}{10}$ of the maximum. The β-enzyme is also present at detectable levels at zero time, and it increases during the increase of DNA synthesis rate, though by a lesser extent than the α-enzyme (namely 7-fold). Contrary to the α-polymerase, it continues to rise also after the DNA synthesis rate has reached a maximum and is in the sharply decreasing phase. The values of the β-enzyme remain thus rather high, at the twelfth day being as high as the α-enzyme, a new finding in human cells. The maximum in β-polymerase activity seems correlated more to the second wave of increase in repair capacity, and should be parallel, judging from the previous experience, to the second wave of DNA enzymes (13). In summary, at late incubation times the α-polymerase is at values much lower than its maximum, whereas the β-enzyme remains at levels close to its maximum.

In order to obtain a better evaluation of the relative levels of the two polymerases, we fractionated the extracts on sucrose gradients to distinguish them also on the basis of the sedimentation properties. The results are reported in Fig. 2. For the interpretation of the patterns it must be borne in mind that the yield of α-polymerase in the gradient is low (30% of the input versus 70% for the β-enzyme) and that the fractions responding positively to the α-assay but corresponding as sedimentation profile to the single peak observed in the β-assay contain in fact the latter enzyme. One can thus observe that the α- and β-enzymes are both rising in the early days with a maximum for the α-polymerase around the sixth day; at later times the proportion of the α-enzyme decreases steadily, whereas, the β-polymerase remains at a substantial level; the maximum for this enzyme is observed also by this procedure at the eighth day, and is not correlated with the DNA replication peak.

These observations are even more clearly exposed in the results obtained in another similar experiment reported in Figs. 3 and 4. This experiment was performed with conditions identical to those of the experiment of Figs. 1 and 2, except that the UV doses were partially different. The peak of DNA replication rate is observed at the fourth day, followed by a sharp decline down to levels of the order of the zero time level; the DNA repair capacity shows again two peaks, the first one of which is 1 day in advance of the DNA replication peak, whereas, the second one occurs 3 or 4 days later than the first one. The variations of the polymerases show that the α-polymerase rises again in parallel to the DNA replication rate, and decreases together with it (Fig. 3). The β-enzyme instead, after a clear but more moderate rise at the same time as the α-polymerase, shows a second increase, reaching values definitely and surprisingly higher than the α-polymerase, with a maximum on the ninth day. The level of the β-polymerase remains high also at later times, when the α-enzyme is down to levels close to the threshold of detectability. For the late days the data of the enzyme activity fractionated on sucrose gradients show the near complete disappearance of the α-enzyme between the seventh and the tenth day, whereas, the level of the β-enzyme is not undergoing other major variations (Fig. 4).

Absence of terminal deoxynucleotidyl transferase

The extracts obtained in the experiments reported in Figs. 1 and 3 were assayed also for terminal deoxynucleotidyl transferase. No activity was observed (i.e., less than 0.01 unit/10^8 cells), whether at zero time or at any stimulation time; this observation is in agreement with the results of other authors for the early stimulation days (17) and shows that the later wave of enzyme increase does not seem to involve the terminal transferase.

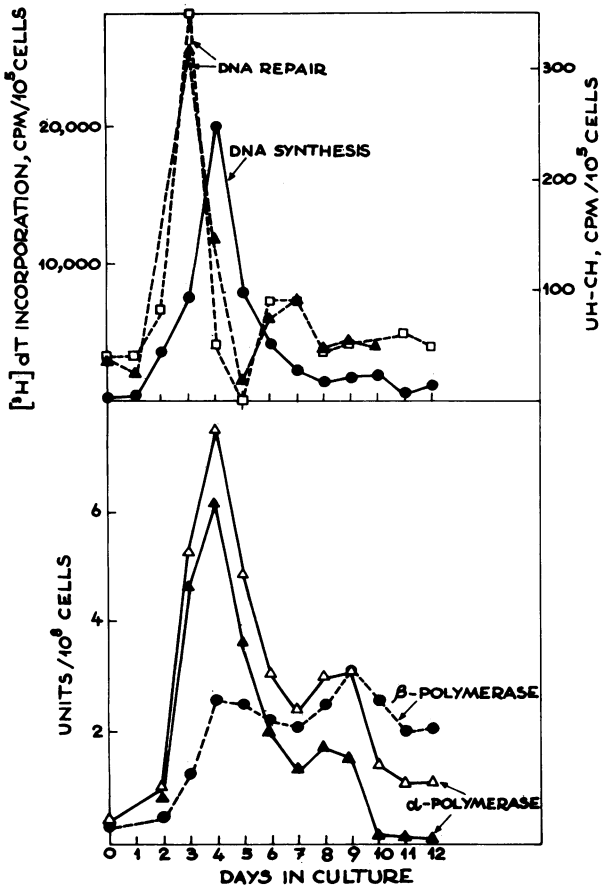

FIG. 3. Variations with time of the two DNA polymerases and of the other parameters during stimulation of human lymphocytes with PHA. The experiment was performed on blood from a different donor, and the procedure was identical to that of the experiment of Fig. 1, except that the doses of UV radiation used to determine repair capacity were 480 erg/mm² (□- - -□) and 720 erg/mm² (▲- - -▲). For other details, see legend to Fig. 1.

DISCUSSION

The experiments reported in this work demonstrate, in the first place, that the DNA polymerases-α and -β are present in the unstimulated lymphocytes, though at a low level; this agrees with the results of other authors (11) and suggests that the inability to observe these enzymes reported in some cases (5, 18) is due to the insufficient sensitivity of the assay. Furthermore, both enzyme levels rise pronouncedly after the stimulation with PHA during the increase in DNA synthesis rate, in agreement with the reports by Coleman *et al.* (11) and by Mayer *et al.* (18).

At late incubation times, after the sixth day of incubation, when DNA synthesis rate is decreasing to values of the order of those of unstimulated lymphocytes, also the α-polymerase decreases, and may reach values lower than at zero time; the β-polymerase instead continues to rise, reaches its maximum levels 3 to 4 days later than the α-enzyme, and remains at levels close to its maximum until the twelfth day.

Thus, the rise in α-polymerase, in agreement with the published data (11, 18), is parallel to the rise in replication rate, whereas the rise in β-polymerase does not parallel the replication rate; the maximum in β-polymerase level is attained in fact at a time when DNA replication rate is minimal. The timing of the β-polymerase maximum seems correlated to the second peak in DNA repair capacity measured in these experiments and corresponds probably to the second wave of increase of other enzymes acting on DNA, observed in our previous work (13).

The measure of UV-stimulated DNA synthesis we have utilized in this work offers a reliable parameter of the ability of the cells to perform repair synthesis, as demonstrated by the fact that cells of patients with xeroderma pigmentosum (whether fibroblasts or unstimulated lymphocytes) show a significant defect in the (UH − CH) values (14, 19), in agreement with the data obtained by more complex procedures. The response elicited by UV radiation in the lymphocytes is not appreciably dose-dependent in the range between 480 and 1080 erg/mm². In fact, in other experiments (L. Dalprà, M. Stefanini, E. Giulotto, A. Falaschi, and F. Nuzzo, manuscript in preparation) four different doses were used in stimulated lymphocytes, and the responses observed in the (UH − CH) values were similar at all the doses. In all the experiments, the capacity of the stimulated lymphocytes to perform repair synthesis follows the same basic pattern, i.e., it reaches a maximum correlated (though not always

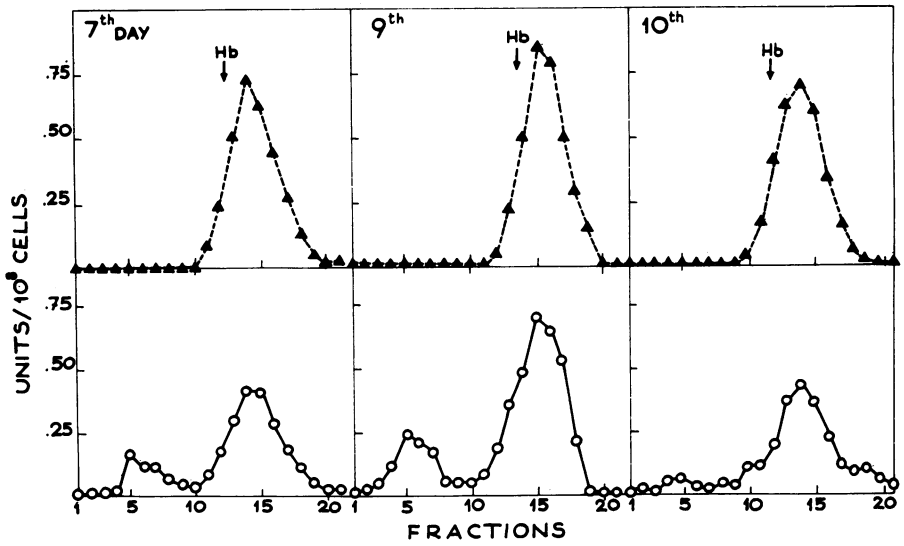

FIG. 4. Sucrose gradient fractionation of α- and β-polymerases at selected days of stimulation. The fractionation was performed on the same extracts obtained for the experiments of Fig. 3. For the details, see legend to Fig. 1. The upper panel reports the absolutely β-specific assay (▲- - -▲); the lower panel, the partially α-specific assay (O—O). Direction of sedimentation is from right to left.

coincident) with the DNA replication rate, and remains at high values also at late times, when DNA replication rate is minimal and a second peak of DNA enzymes is observable.

It must be emphasized that in all the work performed with the PHA-treated lymphocytes a great variability is observed in different experiments as to the time of maximum replication rate, the extent of increase of such rate, and the extent of induction of the different enzymes (13); this variability applies also to the measure of the repair capacity, but, for this as well as for the other parameters just mentioned, the basic pattern of response is quite reproducible.

The data obtained in bacteria indicate a certain specialization of function of the different DNA polymerases with respect to replicative or repair-type synthesis (20). The data we present here suggest also tendency to a partial specialization of functions for the two polymerases of human cells; thus, the α-enzyme is mainly correlated to the chromosome replication process; the β-enzyme level seems more correlated (at least in the lymphocyte cultures) with the attainment in the cell population of a particular condition in which DNA repair capacity is high and DNA replication rate is almost not detectable; this particular cellular state requires also the presence of other enzymes acting on DNA (13).

The nature of such a condition can be object of speculation in many directions, but requires in the first place a description at the molecular level of the possible rearrangements occurring in the lymphocyte DNA.

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