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Levels of some enzymes acting on DNA in xeroderma pigmentosum

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ABSTRACT

We have determined the levels of DNA polymerase, DNA ligase, a DNase acting on single-stranded DNA, an endonuclease making single-strand breaks in double - stranded DNA and polynucleotide kinase in fibroblasts obtained from nine normal persons and from nine patients with Xeroderma Pigmentosum; the pathological lines belong to the different described clinical forms and to the three different comple - mentation groups described so far. All the enzymes are present in the normal lines and in the Xeroderma lines. The levels are quite variable, but the values obtained in the pathological lines lie within the ones observed in the normal population.

INTRODUCTION

The cells of Xeroderma Pigmentosum (X. P.) patients are defective in the process of repair of UV radiation damage to DNA (1, 2, 3, 4). The evidence obtained from some cell lines suggests a defect in one early step of the thymine-dimer excision process, probably the incision step (2, 3, 5, 6). There is ample evidence of heterogeneity in the X. P. cases;the clinical symptoms allow to describe a "classical" form, (distinguished in moderate and severe cases) and the De Sanctis-Cacchione syndrome, where also neurological signs are present. The different cases are also heterogeneous in the degree to which the repair process is reduced: the degree of impairment in the repair synthesis, as determined by the measurement of unscheduled DNA synthesis, or of non-semiconservative replication, is quite variable among the different subjects and can be nil (7). Direct evidence of genetic heterogeneity is obtained by a complementation test in virus - induced cell hybrids (8, 9) and allows to describe at this moment three complementation groups. It is safe to maintain at present that the

X. P. phenotype in man indicates sensitivity to UV (and not χ or γ) radiation, and that a variety of genotypes (at least three) and molecular defects may correspond to it.

It seems highly desirable to obtain direct data on the levels of enzymes probably involved in the DNA repair process in X. P. cells. The task is made difficult by the choice of materials on which to perform the assays. The leukocytes, immediately after isolation, are very low in the enzymes of DNA metabolism. After stimulation of the lymphocytes with phytohemoagglutinin (PHA), the levels of some enzymes acting on DNA raises by a factor of 50 or more over the background levels (10); the PHA stimulated lymphocytes may supply then a source of cells for the enzyme measurements relatively easy to obtain. Another possible source are fibroblasts obtained from skin biopsies and cultivated in vitro; this can never be an abundant source of cells, but may offer advantages in terms of reproducibility. Bacchetti et al. (11) have reported assays in fibroblasts of an endonuclease acting preferably on UV irradiated DNA; no significant reduction was observed in X. P. cells with respect to the controls. We have assayed a number of enzymes of DNA metabolism in leukocytes and in fibroblasts and we will report the results obtained from both types of cells. Preliminary reports of this work have been already published (12, 13, 14).

MATERIALS AND METHODS

Cell sources - The kind of cells used and the subjects from whom they were obtained are reported in Table I. They include nine unrelated controls of different ages. The Xeroderma patients are also nine; they are identified, according to a proposed convention, by the XP prefix followed by a number and by two letters indicating the place where the primary culture was initiated. Thus PV stands for Pavia, RO for Rotterdam, PR for Pavia-Roma.

The cases belong to the two clinically distinguishable forms, classical and De Sanctis-Cacchione. The ability to perform repair replication of the cases from Rotterdam was measured by Klejjer et al. (7); the amount of unscheduled DNA synthesis was reduced with respect to the normal control down to 40% for XP2RO, to 10 - 15% for XP4RO and to less than 5%

Table I

List of sources of lymphocytes and fibroblasts (normal and X.P.)

Denomination	Kind of cells obtained	Sex	Age	Clinical symptoms
I	Lymp.	M	38	Normal
II	Lymp.	M	36	Normal
III	Fibr.	M	13	Normal
IV	Fibr.	F	35	Normal
V	Fibr.	M	phoetus	Normal
VI	Fibr.	F	30	Normal
VII	Fibr.	F	54	Normal
VIII	Fibr.	M	26	Normal
IX	Fibr.	F	23	Normal
XP1PV	Lymp., Fibr.	M	65	Moderate
XP2RO	Fibr.	F	34	Moderate
XP2PV■	Fibr.	F	12	Severe
XP3PV■	Lymp.,Fibr.	F	10	Severe
XP5PV	Fibr.	M	13	Severe
XP4RO	Fibr.	F	16	Severe
XP6PR*	Fibr.	F	15	De Sanctis-Cacchione syndrome
XP7PR*	Fibr.	M	19	"
XP25RO	Fibr.	M	1	"

■ First cousins
* Siblings

for XP25RO. These three cases belong also to the three different complementation groups described by De Weerd-Kastelein et al. (9).

Cell cultures - The preparation of lymphocytes was accomplished from 10 to 20 ml of blood; after 72 h of stimulation with phytohemoagglutinin the cells were collected, extracted and assayed as reported previously (10) for the enzymes indicated below; fibroblast cultures were initiated from

skin biopsies of normal persons and of X.P. patients and maintained in Eagle's medium supplemented with 20% calf serum; the subcultures were made by trypinization (Trypsin-Difco, 1:250, 1 mg/ml); for the enzyme assays we used cultures of different numbers of passages, from three to twenty.

Enzymes assays - Extracts for the enzyme assays were made from approximately 0.1 g (wet weight) of packed fibroblasts in the conditions reported before (10). DNA polymerase was assayed with calf thymus DNA template, activated by pancreatic DNase treatment; DNase acting on single-stranded DNA (possibly corresponding to the DNase III of Lindahl) was assayed as previously reported (10); ligase was assayed using as substrate the interrupted poly(dT)strand, covalently attached to cellulose at the 5'P end, and annealed to poly(dA) (15); polynucleotide kinase was assayed as described by Richardson (16). "Nickase" (that is an endonuclease that makes single strand breaks on double stranded DNA) was assayed on a substrate like the one for ligase, but in which the poly(dT) strand is uninterrupted; an endonucleolytic break on the poly(dT) strand alone may release, in alkali, a poly(dT) chain that can be easily removed from the cellulose and still be long enough to be acid precipitable. The preparation of the substrate, the conditions for the assay and the properties of the main enzyme responsible for this "nickase" activity will be published elsewhere.

RESULTS AND DISCUSSION

Lymphocytes - PHA stimulated lymphocytes contained the amounts of DNA polymerase, ligase and denatured DNA DNase reported in Table II. No appreciable variations are observed; on the other hand, as reported by Pedrini et al., the levels of these enzymes are subject to impressive regulatory variations in stimulated lymphocytes; in fact the time at which the enzymes reach their maximum is different for the different enzymes, polymerase and DNase being at their highest point one day before ligase (10). Furthermore, the maximum of enzyme stimulation is reached at different times in different experiments and the maximum values are quite variable in different normal subjects. All these points raise the possibility that the varia-

Table II

Levels of ligase, DNA polymerase and DNase in stimulated lymphocytes

Each value is the average of two assays in the linear range of activity. The ligase assay contained 0.8 nmol of cellulose poly(dT).poly(dA) where the poly(dT) strand is labeled with tritium at a specific activity of 8.4 cpm/pmol. The DNase assay contained 5 nmol of B. subtilis DNA labeled with tritium, at a specific activity of 9.6 cpm/pmol. All other details as reported by Pedrini et al. (10).

Case	Clinical symptoms	Ligase u/mg	DNA polymerase u/mg	Den. DNA DNase u/mg
I	Normal control	1.7	0.16	5.2
II	Normal control	1.7	0.14	7.5
XP1PV	Moderate	0.93	-	6.3
XP3PV	Severe	1.1	0.085	1.2

tions of enzyme levels due to the massive regulatory phenomena may mask and overwhelm any inherent difference in the level of the enzymes under study. We thought therefore that a better source may be given by the fibroblast cultures.

Fibroblasts - The activities of the enzymes under study can be revealed in the fibroblast cultures, though at a lower level than at the peak of stimulation lymphocytes. Table III reports the data obtained from two X.P. cases and four different controls using the same substrates as for the experiments with lymphocytes. The levels of polymerase and ligase were lower than in the lymphocytes; ligase, in fact, was below the background level and we were able to assay it only by raising the cellulose concentration by a factor of 5, so that the ligase values are not directly comparable to those of the lymphocytes, since we are always in limiting concentrations of substrate. The ligase levels (expressed as units/mg), assayed in similar

Table III

Levels of DNA enzymes in fibroblast lines

(First batch of reagents)

Each value is the average of at least three experiments, each consisting of two assays in the linear activity range. Ligase assays contained 4 nmol of the same preparation of substrate as for the experiments of Table I. Endonuclease assays contained 25 nmol of cellulose poly(dT).poly(dA), where the poly(dT) was labelled with tritium, at a specific activity of 3.1 cpm/nmol. All other details as in Table II.

Case	Clinical symptoms	Ligase u/mg	DNA polymerase u/mg	Den. DNA DNase u/mg	Endonuclease u/mg
III	Normal control	0.30	0.067	4.9	-
IV	"	0.13	0.024	3.1	13
V	"	-	-	1.4	20
VII	"	-	0.018	-	-
XP1PV	Moderate	0.074	0.034	4.9	17
XP2PV	Severe	0.038	0.039	3.0	5.0

conditions, are between 3 and 10% of the values obtained from established cell lines (15) and from stimulated lymphocytes at the ligase peak.

The data are quite reproducible; in fact no appreciable variations are observed if the extract is made out of fibroblasts from the same cell line at different numbers of passages. Thus, e.g. line XP1PV assayed after three, nine and ten passages gave values of polymerase of respectively 0.029, 0.018 and 0.024 u/mg; line XP2PV after seven and nine passages gave values of ligase of respectively 0.033 and 0.032 u/mg. All the variability seems to be among the lines. The data of Table III show consistently reduced levels of ligase only in the X.P. lines. The doubt arises whether the observed reduction, down to 12% for XP2PV vs control III, represents a significant deficiency of the Xeroderma lines with respect to the amount

of ligase needed for physiological growth and survival in the normal population. We decided therefore to extend the number of control lines and X. P. cases to further increase the sensitivity of the assay by preparing new substrates. The new set of data is reported in Table IV. The observed apparent reduction in ligase of XP2PV vs the control lines is still observed: XP2PV has about 15% of the ligase activity with respect to control IX; on the other hand, a new control, line VI, has ligase values of the order of those of XP2PV and XP1PV, i.e. of the lowest ones observed in X. P.

It would seem then that the variability in the normal population is quite pronounced and that it may reach tails with values of the order of those observed in the X. P. cases with the lowest enzyme levels. Similar considerations can be made from the other enzymes tested. In all cases the variability is very pronounced in all the lines tested, but the values of the pathological lines lie within those of the control lines. There is no correlation with the age of the culture or with the number of passages; there is also no correlation with the degree of impairment in repair capacity, since the lines include all the different degrees of impairment, as reported in "Materials and Methods". The variability seems to be mainly among lines, and the same lines, extracted at different culture times, give similar values.

The conclusions that can be drawn from this work are the following:

1) The levels of DNA polymerase, ligase, DNase, nickase and polynucleotide kinase are quite variable in fibroblasts from normal subjects; the variability is not surprising and is observed also in the most abundant enzymes from standard sources.
2) The same enzymes are all present in the cells of X. P. patients, belonging to all the clinically distinguishable forms and to all the three complementation groups described so far.

The observation of the presence of the enzymes in question may mean:

a) that the defect in the X. P. lines lie elsewhere, like, e. g. in a UV specific endonuclease, as described for the uvr mutants of Escherichia coli and Micrococcus luteus (17, 18).

Table IV

Levels of DNA enzymes in fibroblasts

(New batch of reagents)

Each value is the average of two assays in the linear activity range. Ligase assays contained 13 nmol of substrate with a specific activity of 25 cpm/pmol. DNase assays contained 5 nmol of substrate with a specific activity of 7.7 cpm/pmol. Endonuclease assays contained 18 nmol of tritiated substrate, with a specific activity of 1.3 cpm/pmol.

Line	Clinical symptoms	Ligase u/mg	DNA polymerase u/mg	Den. DNA DNase u/mg	Endo-nuclease u/mg	Kinase u/mg
VI	Control	1.4	0.036	8.9	3.9	0.24
VII	"	3.7	0.20	6.4	7.1	-
VIII	"	4.9	0.74	2.5	3.5	0.20
IX	"	5.8	1.32	5.3	7.7	0.20
XP1PV	Moderate	1.1	0.079	4.9	6.8	0.11
XP2RO	Moderate	3.9	0.38	1.8	-	-
XP2PV■	Severe	0.9	0.095	5.7	1.8	0.13
XP3PV■	Severe	4.8	0.11	6.1	2.9	0.20
XP5PV	Severe	3.7	0.29	1.8	3.8	0.24
XP4RO	Severe	2.5	0.97	6.4	2.5	0.10
XP6PR*	De Sanctis Cacchione	5.1	0.83	3.6	-	-
XP7PR*	De Sanctis Cacchione	4.7	1.2	4.3	-	-
XP25RO	De Sanctis Cacchione	4.5	0.89	4.1	-	-

■ First cousins
* Siblings

b) that the enzymes we assay are in fact multiple (as demonstrated for DNA polymerase, and most likely for the DNases) and an underlying defect may be masked by the presence of another enzyme responding to the same assay.

This requires that the investigation be extended to include assays of other enzymes, and that the techniques be established to determine quantitatively the relative amounts of enzymes with similar catalytic properties but different functions inside the cells. Fibroblast cultures still seem to be the most advisable source of DNA enzymes in man for studies of this kind since the stimulated lymphocytes undergo too great regulatory variations.

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