A Progress Report
to
U.S. ENERGY RESEARCH AND DEVELOPMENT ADMINISTRATION

BASIC ASPECTS OF
RADIATION ACTION ON MICROORGANISMS

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Progress Report

The basic character of the project has been the study of the process of induction of virus-like bodies in cells. For this purpose the model system of Escherichia coli has been chosen.

Relation of work to the mission of ERDA

If there is to be any use of nuclear energy on any large scale then the hazards that result from it have to be carefully assessed. The underlying reason for this work in relation to ERDA is that it will give suggestions for the mechanism of carcinogenesis and mutagenesis by ionizing radiation. In both these cases in the past rather long extrapolations have had to be made between work in which definite conclusions regarding these phenomena can be made and the actual levels which are probably encountered by the population. If there is some idea of the mechanism by which these phenomena occur, the extrapolation becomes much more reliable. One important suggestion that has come out of work in the past year has been that some mutagenesis does not occur by a direct linear mechanism but instead involves a two-fold process. One part of the process is the induction by damage to the DNA of the cell of an error-prone repair system (erpr) and the second part is that mutations result from the operation of the erpr. This strongly suggests that low background radiation has little or no effect in producing mutations. It further suggests that single exposures to moderate or high levels of radiation may conceivably put the individual at risk for the rest of his life (see Appendix 1).

Outline of past history of the project

The study of induction began with early work on λ prophage. Since then it has been recognized that there are genetic factors which greatly influence the inducibility of prophage. For example the various rec mutations, the lex mutation, and perhaps most importantly, the discovery of the temperature inducibility gene tif. These factors have shown that λ is very difficult to induce when the recA and lex genes are lacking and that it is temperature inducible in cells carrying tif. At the same time a number of phenomena have been found which also depend on the presence of these genes. The list of these phenomena, including the induction of λ , is given in Table I, and

for all these phenomena a striking difference is observed if the cells are lacking in the recA and lex genes and if they are at the wrong temperature for the tif gene.

TABLE I
Induced Error-Prone Repair System: Manifestations

-
- ^a Induction of prophage
 - ^a UV reactivation and mutagenesis
 - Filamentation
 - ^b Protein X
 - Protein G - septum involved
 - ^a Error-prone mutagenesis
 - ^b Induced inhibition of postirradiation DNA degradation
 - ^a Induced radioresistance
 - ^b Induced single-strand break repair system
 - Induced respiration halt.
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^a Under study at Duke University and The Pennsylvania State University

^b Being studied at Penn State.

Our laboratory became involved in this line of research through work by Pollard and Randall (Radiat. Res. 55:265, 1973), which was supported by this grant, in which the inducibility of the inhibition of DNA degradation was shown. This was further related to recA and lex genes by Marsden, Pollard, Ginoza and Randall (J. Bact. 118:465, 1974). The relationship to the repair of single strand breaks was further shown by Tolun, Christensen and Pollard (Biophys. J. 14:691, 1974), also supported by this grant. More recently, reported late last year (Pollard and Achey, Biophys. J. 15:1141, 1975), was the discovery of induced radioresistance, which is also one of these phenomena, done collaboratively at the University of Florida (Gainesville). During the past year the induction of erpr mutagenesis by ionizing radiation has been shown and reported by Pollard, Person and Rader (in press, Radiat. Res.).

Personal factors in this report

A short statement of the nature of the contribution which can be made by the principal investigator is in order. As a relatively older citizen still active in research, we have much less inhibition on collaboration than is normally the case with an individual who is tied down to teaching and other responsibilities. Accordingly, I have collaborated at the University of Florida, largely with Dr. P. Achey, and this past winter at Duke University with Dr. D. J. Fluke. The purpose of the latter collaboration has been to exploit a very fine UV monochromator (constructed by Dr. Fluke) for examining the wavelength dependence of much of this whole range of inducible phenomena. The preliminary work on this has been completed this past winter and will be discussed later. Therefore there will be in future requests for support a clear dichotomy. Part of the time effective work can be done collaboratively elsewhere and part of the time continuing work in a laboratory at Penn State should be carried on. In order to make this work effective the laboratory effort in both institutions has a second member understanding the work and capable of carrying it on. At Penn State this is Dr. S. Person and at Duke U. it is Dr. D. J. Fluke.

Present state of understanding of the induction process

A very compact way of indicating the nature of the induction process has been given by Lorraine Gudas and Arthur Pardee (1975). This is indicated in Fig. 1 and is to a large extent self-explanatory although several items arise which need further work. The significance of the process is that some class of damage to the DNA of the cell, damage often associated with ionizing radiation and UV, causes the derepression of an operon and the transcription and translation of proteins involved in response to the radiation damage. At least four functions can be claimed for the proteins: inhibition of DNA degradation, mutagenesis at gaps involving dimers, increased repair of single-strand breaks, and a cessation of respiration. In addition, the transcription of this operon is involved in some way with the induction of prophage. The understanding of the process is therefore of central importance to the understanding of the biological action of radiation. To the already known and partly catalogued mechanisms of repair we must now add a multiply acting and induced system, which not only is effective in providing resistance against

Fig. 1. The scheme of Cudas & Pardee for the induction by radiation or other damage to DNA of the error-prone system. This is supposed to be controlled by the tif gene, which requires to lose repression before the proteins of the erpr system can be transcribed and translated. In wild type cells the tif is repressed by a repressor synthesized in response to the lex and tsl genes. These give a protein repressor which binds very firmly to the DNA at the tif region and prevents transcription. If the cell is normal, the recA gene is present and its gene product is a protein molecule that can combine with the repressor and render it sensitive to a second effector molecule. The nature of this effector molecule is not finally known, but evidence implicates breakdown products of DNA which follow various treatments as listed in the diagram. The combined action causes derepression of the tif operon with the result that the pleiotropic response of the operon is found. Protein X, which binds to single-stranded DNA is one such, erpr mutagenesis is another, and respiration halt is a third.

radiation, while increasing the mutational probability, but is ephemeral in character, in that the inducing signal will not persist in future generations. All of the essential characteristics of this important and newly discovered system need to be described and understood. The work on this grant is now wholly engaged in playing a part in such description and understanding.

To return to the specific suggestions of Gudas and Pardee, first, of course, any such scheme as this requires definitive proof. It also requires a characterization. One feature that is not yet clarified is the nature of the signal which causes the induction of the tif operon. In the scheme of Gudas and Pardee the oligonucleotides produced by the break-up of DNA furnish the signal. An alternative suggested from our laboratory is that the secondary structure of the nuclear body of the bacterium can be locally disrupted and this can be the signal. It is also necessary to establish whether the induced part is one single protein with multiple functions or whether there are several proteins.

Work in our laboratory has been concerned with these additional requirements of proof and elaboration. We have some unreported experiments done at Gainesville in which attempts were made to introduce nucleotides into calcium-treated cells, which are more penetrable than are normal cells. These results were all negative and as far as I know similar experiments done elsewhere were also negative. This is not disproof of the hypothesis of Gudas and Pardee; rather it merely suggests that it is hard to do, especially treated cells in this kind of study.

We have also looked at the induced part to see whether it is just one protein. We are strongly doubtful of this. One approach, taken in the work at Penn State, has been to see whether or not there is complete agreement between the kinetics of induction of the inducible inhibitor of post-irradiation degradation and the induced repair of single-strand breaks. There seems to be a likelihood that in normal cells there is a separation between the two. That is to say, we can observe quantitatively a certain measure of inhibition of DNA degradation and this does not match the amount of repair observed. We have also examined dose-response curves to see if differences exist. Figure 2 shows four dose-response curves of which two

Fig. 2. Four dose-effect relationships for various inducible factors. The three that apply to strain AB1157 seem to go together, although additional work is needed on the single-strand break repair. On the other hand, the introduction of λ into strain AB1157 markedly changes the nature of the fluence needed to produce an effect. There is no radioresistance observable at all, and the induction of λ requires higher fluences by a factor near 10.

are pretty tentative (induction of single-strand break repair and induction of λ). There is a clear difference between the dose-response curve for the induction of λ and those for the other phenomena. The high dose necessary to induce λ is found for both UV (recent work done with Dr. Fluke) and for ionizing radiation (Lydersen and Pollard, 1975, previous work done on this grant). We are therefore quite sure there is a separation with regards to the induction of λ . We think it probable that there is a separation between the inhibition of DNA degradation, of induced radioresistance, the production of induced single-strand break repair on the one hand, and the induction of protein X on the other.

Induced repair of single-strand breaks

The increased repair of single-strand breaks after treatment with UV and then incubation was shown by Tolun, Christensen and Pollard (1974). We have followed this up by a modification along the lines of the work on induced radioresistance. The procedure is indicated in Fig. 3. Varied doses of UV are given to cells labeled in their DNA with ^3H -thymidine. The cells are then incubated with aeration at 37°C for 40 min, the standard time allowed for the induction system to develop. They are then treated with rifampin at 50 $\mu\text{g}/\text{ml}$ for 10 min at 37°C and given 39 krads X-rays, sufficient to produce a good many single-strand breaks. One batch of the cells is immediately chilled in preparation to lysing for gradient studies, and another is incubated at 37° for 20 min to allow repair to take place.

The cells are then lysed on top of an alkaline sucrose gradient of the familiar type using 5-20% sucrose and spun for about 100 min in the ultracentrifuge. Fractions are collected onto filter papers which are then suspended in 5% trichloroacetic acid. After this they are placed in scintillation vials and counted. The counts observed in each fraction are then plotted directly in the figure (Fig. 3). It can be seen that the 20-min incubation period causes an increase in the size of the DNA, meaning the repair of single-strand breaks. As the pre-dose of UV is increased the amount repaired in this time increases up to about 60% as estimated by the relative number of counts in the repaired peak divided by the original peak. Quite low doses of UV will elicit the additional repair and in

Fig. 3. Repair of single-strand breaks after previous doses of UV. The technique of alkaline sucrose gradient study is used to observe the size of the DNA. The counts in the fractions with the top at the left are shown; the solid lines are for cells that received UV, were incubated 40 min, given rifampin at 50 $\mu\text{g/ml}$, and then exposed to 39 krad X-rays. The DNA is fragmented and appears spread out in a broad band. If 20 min is allowed at 37° for repair to take place, the size of the DNA is seen to increase. If the pre-treatment is a low fluence the degree of repair is less; at higher pre-treatment fluences there is considerable repair (lower panel). In Fig. 2 the results of several such experiments are analyzed to give a dose-response relation for induced repair; these are only preliminary, but not out of line with other induced phenomena.

Fig. 2 are shown three dose-response curves for strain AB1157. These are for the induced single-strand break repair, for which only preliminary data are available and which is the most demanding on technique. The second is for induced radioresistance, and the third for induced inhibition of post-irradiation DNA degradation.

Induced radioresistance

This phenomenon has been described by Pollard and Achey (1975) and has also been seen by Smith and Martignoni (in process of publication). Our procedure is as follows. The survival of colony-forming ability under treatment by X-rays is used as a test of radioresistance. Since X-rays themselves induce the error-prone repair system it is necessary to prevent the effect of such induction from showing in the survival curves. Accordingly, before X-ray is given to the cells they are treated with rifampin, which blocks transcription and prevents the formation of the proteins involved in the *erpr* system. Comparison is made between cells induced for *erpr* by a dose of UV, followed by incubation to permit transcription and translation to occur, and non-induced cells. The rifampin treatment is applied to both. The time-flow chart of an experiment is shown on Fig. 4 in which cells of AB1157, a strain of K12 which is not deficient in any of the radiation-response genes (i.e. it is *recA*⁺, *uvr*⁺, *lex*⁺) is studied. The survival curve for the non-pretreated (and so non-induced) cells is on the left. On the far right is the survival curve for cells which received a surface dose of 265 nm UV of 19.96 J/m^2 , were incubated for 40 min and then X-irradiated. Those receiving 3.85 J/m^2 of the same wavelength light gave the second line from the right, and if 365 nm light, at $1,059 \text{ J/m}^2$, is used the line closest to the non-pretreated case is found. Obviously there is considerable radioresistance developed by pretreatment at 265 nm, and doubtful radioresistance at 365 nm.

By plotting a dose-response curve at 265 nm and comparing the dose to produce a given degree of radioresistance at some other wavelength with that at 265 nm an action spectrum can be derived, which is given in Appendix II.

Effect of introducing λ into AB1157

Dr. R. Boyce (U. Florida) kindly sent us cells of AB1157 which had been lysogenized by λ . These were used to test radioresistance with the results

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Fig. 4.. The phenomenon of induced radioresistance in strain AB1157. With no UV pre-treatment the cells are given rifampin at 50 μ g/ml, allowed to incubate for 10 min at 37° for the rifampin to take effect, and then given the doses of X-rays shown. If a predose of UV as indicated at the wavelengths indicated is given, and then following incubation for the translation of the induced proteins the cells are given rifampin as before and irradiated with X-rays, the sensitivity is markedly less, as seen. Pretreatment with a considerable fluence of 365 nm light is not very effective in causing radioresistance.

shown in Fig. 5. This is one of three experiments. It is our conclusion that the introduction of λ into the genome not only drastically alters the induction dose-response curve, as can be seen in Fig. 2, but also removes one of the induced properties. The predose was varied from 4 J/m^2 to 24 J/m^2 with no effect observable.

A similar result has been found with strain 15, which carries a defective prophage. J. Fugate, at Penn State, found that this strain did not show radioresistance, even when cured of the "colicinogenic factor". Presumably the curing process has rendered the strain effectively lex⁻, since the degree of postirradiation DNA degradation is very great and there is no inducible inhibitor. Therefore, in two strains we have evidence that inducible properties are drastically altered if there is an inducible prophage on the genome.

Dose effect curves for uvr⁻ induction of radioresistance

Recently, in collaboration with Dr. D. J. Fluke, a dose-effect curve for induction of radioresistance in strain WU3610-89 has been obtained. In this series of experiments attention was paid to proper measurement of the average fluence as regards depth. This was done by using a silica cell for exposure and monitoring the light on the surface and transmitted. The data for the lateral averaging across the spectral line are taken but the computations are not yet done. The estimate is an average of 65%. The remarkable finding is that in this uvr⁻ strain the full effect of radioresistance is attained at 0.2 J/m^2 . Allowing for the lateral averaging, this drops to 1.35 ergs/mm^2 , meaning that about 10 dimers per genome will cause induction. This is so small a number that it puts sharply in question the idea of oligonucleotides acting biochemically as the inducing agent. The maximum number of oligonucleotides per cell would be less than 50 and this is a very low concentration. More attention paid to these sensitive inducing systems might elucidate the induction process.

Action spectra

In the collaborative work with Dr. D. J. Fluke which has been the subject of the efforts this fall and winter we have covered in a preliminary way the UV action spectra for the induction of λ , the induction of radioresistance,

Fig. 5. The failure of lysogenized AB1157 cells to show radio-resistance. The cells which have received 10 J/m^2 predose are actually more sensitive, if anything.

mutagenesis, the part of the killing of strain B_{s-1} with and without photoreactivation, and the killing of the uvr⁻ strain with and without rifampin.

Some of this work was supported by a now expired contract with the Bureau of Radiological Health, but most of the work has been part of this ERDA grant. The various action spectra are shown in Appendix 2, and it is interesting to note that while they broadly confirm the concept that most of the effect matches the absorption of the DNA there are still marked deviations. Three deviations can be mentioned: one, the increased level of killing at 280-300 nm, which is also found by others (e.g., work by R. Setlow, 1974); secondly, the relatively high efficiency of mutagenesis at 280-300 nm; and third, the killing efficiency of the short wavelength region appears to be less.

SUMMARY

The work in the last year has shown the following major things:

1. Error-prone repair mutagenesis can be induced by ionizing radiation (and UV light).
2. Induced single-strand break repair has been confirmed and its characterization is in process.
3. There is clearly a separation between the induction of λ and other phenomena. The dose-effect relationship differs and the phenomenon of radioresistance is removed.
4. Action spectra for UV mutagenesis and the killing of B_{s-1} with and without PHTR have been plotted.

Literature Cited:

- Gudas and Pardee, PNAS 72:2330-2334, 1975
Setlow, PNAS 71:3363-3366, 1974