Bleomycin – Induced DNA Damage and DNA Repair in Chicken Embryo Cells as Compared to X-Irradiation

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Following *in vitro*- and *in ovo*-exposure of chicken embryo cells, the level of bleomycin (BM) – induced damage was evaluated by using DNA synthesis, nucleoid sedimentation (SED), and viscometry of alkaline cell lysates (VISC). This damage was compared to X-irradiation, using 5.9–378 nM BM *in vitro*, 1.5–116 μg BM/egg *in ovo*, and 2–32 Gy, respectively, *in vitro* as well as *in ovo*. With respect to BM, the most notable result is the increase in DNA synthesis and VISC at the lowest concentrations of the drug. A decrease in both parameters was observed at high BM concentrations and following exposure to X-rays, concomitantly with an increase in SED. Regarding the radiomimetic drug BM and X-rays, different modes of DNA damage and DNA repair are suggested by previous investigations and the present results. Therefore, further evidence is presented, that the chicken embryo can act as a simple, rapid and inexpensive test system to characterize the biological effects of many nucleo- and/or cytotoxic agents.

Introduction

In screening for toxicity, the use of the chicken embryo has retained considerable interest, especially as an alternative to experiments with living animals. Comprising a broad range of morphological, biochemical, and functional parameters, the majority of test results correlate with findings in mammalian systems (see, e.g., Kemper and Luepke, 1986; Nishigori *et al.,* 1992; Kucera *et al.,* 1993). For instance, by determining the formation of micronuclei, even genotoxicity testing is possible (Wolf and Luepke, 1997).

In previous investigations, the effects of X-irradiation on the chicken embryo were tested by physico-chemical, biochemical, and morphological methods (Stammberger *et al.,* 1989; Tempel *et al.,* 1995). The aim of our present study was to characterize, by the same tests as used earlier, the influence of bleomycin (BM), the substance being considered as a classical radiomimetic agent and because of its widespread use in the treatment of cancer (see, e.g., Burger, 1998; Huang and Lown, 1996). It will be shown that BM acts upon chicken embryo cells at remarkably low concentrations and that considerable differences exist between the radiomimetic drug and X-irradiation with special reference to DNA repair.

Materials and Methods

Chemicals

[^{3}H]Methyl-thymidine (dT-^{3}H, specific activity 3.33 TBq/mmol) was a NEN$^{(R)}$-product (NEN Life Science Products, Boston, MA, USA). BM was a gift from Mack (Illertissen, Germany). The other test substances and chemicals were from Merck (Darmstadt, Germany), Serva (Heidelberg, Germany), and Sigma (München, Germany).

Cells

Fertile eggs from White Leghorn chickens from a conventional breeding were used. The embryos were removed from the eggs and decapitated at 15 days after the beginning of incubation (37.8 °C, 60% humidity, the eggs automatically rotated six times a day). Liver-and brain cells were prepared as described previously. Liver tissue solution was performed by collagenase (0.1% in Hank's solution: 136.9 mM NaCl, 0.33 mM Na_2HPO_4, 5.36 mM KCl, 0.36 mM KH_2PO_4, 4.18 mM $NaHCO_3$, 5.55 mM glucose, pH 7.3) (for further details see, e.g., Ignatius *et al.,* 1992). The cell suspensions were adjusted to final concentrations of about 10^6-10^7 cells/ml Hank's medium. Microscopically the liver-cell suspensions showed a rather uniform pic-

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ture, whereas the brain-cell preparations, as expected, comprised of many cell types which were not further identified. Cell viability was determined by trypan blue exclusion. After 60 min incubation at 37 °C, e.g., 80±6% of the chicken embryo liver-cells were intact.

Treatment of the cells

To test BM, stock solutions of the substance were prepared in Hank's solution (in general, 2.8 mg of the biologically active substance in 10 ml Hank's solution). 1.0 ml aliquots of the drug were stored at −18 °C until required for the experiment. When testing BM *in vitro*, 0.1 ml of the appropriately diluted stock solutions were added to 1.0 ml of the cell suspensions. The addition of BM was followed by a 30-min incubation of the cells at 37 °C prior to analytical procedures. When testing BM *in vivo* (*in ovo*), 200 µl of the BM solution at the concentration needed were injected onto the inner egg shell. The controls received 200 µl Hank's solution. 0–48 hours following administration of the drug, liver- and brain-cells were obtained as described above.

For X-irradiation of the cells the Kristalloflex 710H X-ray machine (Siemens, Erlangen, Germany) was used (55 kV, 40 mA, half value layer 0.16 mm Cu, filtration with 0.16 mmCu) at a dose rate of 1 Gy/min (calibrated with a Siemens dosimeter in the center of the X-ray beam). During exposure the cell suspensions remained within micro test tubes placed on ice at the beginning of irradiation. The control preparations were handled in the same manner but without irradiation.

Analytical procedures

The analytical procedures comprised physico-chemical and bio-chemical methods. The former ones consisted of nucleoid sedimentation (SED) and viscometry of alkaline cell lysates (VISC), the biochemical tests were scheduled (SDS) and unscheduled (UDS) DNA synthesis.

The nucleoid sedimentation technique (Cook and Brazell, 1975; Cook and Brazell, 1976; Mattern *et al.*, 1987) was performed as described previously (Ignatius *et al.*, 1994). The viscometry of alkaline cell lysates (Marshall and Ralph, 1982) was also described in a previous paper (Tempel and Schleifer, 1995). Scheduled DNA synthesis was measured *in vitro* by the incorporation of dT-^{3}H into the perchloric acid precipitate (6%) of the cell suspensions (for details see, e.g., Ignatius *et al.*, 1994). To determine unscheduled (repair-induced) DNA synthesis, scheduled DNA synthesis was suppressed by hydroxyurea (0.1 M) which was added to the Hank's medium during the cell preparation and the following incubations.

Unless otherwise stated, each data point represents the mean of at least three independent experiments (± the standard deviations). The significance was tested by Student's t-test.

Results

In a first series of experiments, the effects of bleomycin (BM) and X-irradiation on chicken embryo cells *in vitro* were compared by using viscometry of alkaline cell lysates (VISC), nucleoid sedimentation (SED), and scheduled DNA synthesis (SDS). Both, BM and X-rays, caused a reduction of VISC, the reduction being dose-dependent within a range of about 24 to 380 nM BM and 2–32 Gy, respectively (Tables I and II).

ED_{50}-values, i.e., the concentrations diminishing VISC by approximately 50 per cent, were 176 nM BM and 8 Gy, respectively. At BM concentrations of ~6 nM a slight increase in VISC was observed.

Table I. Viscosity of alkaline lysates (VISC) and nucleoid sedimentation (SED) of chicken embryo brain cells immediately following *in vitro* – exposure (30 min, 37 °C) to bleomycin (BM). Mean values given in per cent of the sham-exposed controls (± standard deviation). Control values: VISC: 13.2±2.9s (= elution time through a 6 cm capillary with a diameter of 0.8 cm), SED: 1.4±0.1 cm/2 h, protein content of the lysates 1.2±0.2 mg/ml.

BM	5.9	11.8	23.6	47.2	94.4	188.8	377.6	nM
VISC	116 ±4	102* ±16	90* ±20	76 ±14	62 ±8	46 ±10	33 ±13	per cent
SED	93 ±4	95* ±6	100* ±5	124 ±7	130 ±5	168 ±8	220 ±12	per cent

* $p \geq 0.05$ (not significantly different from the controls).

Table II. Viscosity of alkaline lysates (VISC) and nucleoid sedimentation (SED) of chicken embryo brain cells immediately following *in vitro* – exposure to X-rays. Mean values given in per cent of the sham-exposed controls (± standard deviation).
For control values see under Table I.

X-rays	2	4	8	16	32	Gy
VISC	78 ±11	68 ±10	55 ±8	30 ±5	19 ±4	per cent
SED	83 ±11	80 ±7	66 ±7	74 ±4	88* ±13	per cent

* $p \geq 0.05$ (not significantly different from the controls).

At BM concentrations of ≥ 47 nM SED increased in a dose-dependent manner to almost 220 per cent at the highest BM-concentration used (377 nM) (Table I). A slight, though not significant, decrease in SED was evident at approximately 6 and 12 nM BM (Table I). As compared to BM, the behaviour of SED following X-irradiation was different (Table II). Concomitantly to the decrease in VISC, there was a reduction in SED as well by almost 35 per cent at 8 Gy. With higher doses, SED increased again in such a manner that, at ≥ 32 Gy, no significant differences existed between controls and X-irradiated cells (Table II)

SDS decreased in BM-treated and X-irradiated cells at doses of ≥ 7 mM BM or ≥ 8 Gy, respectively (Table III), the corresponding ED_{50}-values being about 700 mM BM or 32 Gy. With respect to the ED_{50}-values as evidenced by VISC (Tables I and II), doses needed to inhibit SDS were higher, therefore, by more than three orders of magnitude in BM-exposed cells and only about fourfold higher in X-irradiated cells. Therefore, under the present conditions, SDS seemed to be very insensitive to BM as compared to X-rays, no consistent differences being between brain- and liver-cells (results not given in detail).

In a second series of experiments, chicken embryo cells were treated *in vivo* (*in ovo*). As the effects of X-irradiation of the chicken embryo *in ovo* were studied in previous investigations (Stammberger *et al.*, 1989; Tempel *et al.*, 1992; Tempel *et al.*, 1995), the present experiments were limited to BM. In preliminary studies, the drug was given at doses of 1.45–116 µg BM/egg. Provided that the BM dose as a whole is transfered, via the chorioallantoic vascular area, to the chicken embryo, this corresponds to a BM dose of approximately 0,060 to 9.28 µg/g embryo. Within a short interval, i.e., 1.5–3 hours, and 24 hours following exposure, the influence of the drug on brain-cells was tested by VISC, SED, and SDS. With regard to the extremely low viscosity of the lysates formed by the liver-cell preparations, the analysis in these cells were limited to SED and SDS. Consistent and significant results were obtained 24 h following administration of BM (Table IV). At the lowest BM-dose used, the viscosity of alkaline brain cell lysates surpassed the control values significantly. Within the higher dose-range, i.e., at 29, 58, and 116 µg BM/egg, however, VISC dropped dose-dependently. Concomitantly to the fall of VISC, an increase in SED could be observed. With respect to SDS, the stimulation of dT-^{3}H incorporation at BM-doses of < 29 µg/egg was very noticable. Both, VISC and SDS, surpassed therefore the control values at low BM-doses and showed the tendency to decrease within the higher dose-range (Table IV). As far as investigated, liver-cells reacted to BM exposure *in ovo* in an

Table III. Scheduled DNA synthesis (SDS) of chicken embryo brain cells immediately following *in vitro* – exposure to bleomycin (BM) and X-rays. Mean values given in per cent of the sham-exposed controls (± standard deviation). Control values: $(1.1 \pm 0.2) \times 10^6$ dpm/µg DNA.

BM	0.007	0.07	0.7	7.0	70.0	700.0	mM
SDS	138 ±10	118* ±9	100* ±8	90* ±12	67 ±8	50 ±5	per cent
X-rays	2	4	8	16	32	64	Gy
SDS	n.d**	101* ±8	85 ±8	68 ±6	53 ±9	35 ±6	per cent

* $p > 0.05$ (not significantly different from the controls).
** not determined.

Table IV. Viscosity of alkaline lysates (VISC), nucleoid sedimentation (SED), and scheduled DNA synthesis (SDS) of chicken embryo brain cells 24 hours following *in ovo* – administration of bleomycin (BM). Mean values given in per cent of the sham-exposed controls (± standard deviation). For control values see under Tables I and III.

BM	1.45	2.90	29.0	58.0	116.0	µg/egg
VISC	130 ±17	107* ±17	74 ±8	68 ±6	42 ±8	per cent
SED	96* ±8	98* ±7	110* ±4	121 ±5	130 ±8	per cent
SDS	146 ±17	131 ±10	97* ±12	87 ±10	76 ±11	per cent

* $p \geq 0.05$ (not significantly different from the controls).

analogous manner (results not presented in detail).

With regard to the apparently stimulating effects of BM at low doses (Table IV), VISC and SDS were followed in brain-cells 1.5–48 hours after exposure of the embryos to the drug at a dose of 1.45 µg/egg. The results are presented in Table V. Irrespective of the high standard deviations, it is evident that low BM-doses, e.g., 1.45 µg/ egg, are able to elevate, within a period of approximately 12–36 hours after exposure, VISC as well as SDS (Table V). As compared to brain-cells, nearly the same results could be obtained in liver-cells (not given in detail).

Unscheduled DNA synthesis was determined 24 hours after the administration of BM at a dose of 1.45 µg/egg. UDS increased by about 50 per cent in liver-cells, whereas brain-cells remained without significant effects (no detailed presentation). When unscheduled dT-^{3}H incorporation was studied *in vitro*, however, BM concentrations as low as 1 µg/ml were able to stimulate UDS by about 100 per cent, whereas X-irradiation at doses of more than 50 Gy was needed to increase UDS significantly by about 20 per cent (Table VI).

Very similar results were obtained previously by determining poly(ADP-ribose)polymerase-activity of chicken embryo cells exposed to various nucleotoxic agents including BM and X-rays (Ignatius *et al.*, 1992) (Table VI).

Table V. Viscosity of alkaline lysates (VISC) and scheduled DNA synthesis (SDS) of chicken embryo brain cells 1.5–48 hours following *in ovo* – administration of bleomycin (BM) at a dose of 1.45 µg/egg. Mean values given in per cent of the sham-exposed controls (± standard deviation). For control values see under Tables I and III.

Time	1.5	3	6	12	16	24	36	48	hours
VISC	97* ±4	104* ±11	93* ±21	138 ±14	146 ±16	130 ±17	135 ±9	100* ±12	per cent
SDS	103* ±10	111* ±17	132 ±7	164 ±20	154 ±10	160 ±20	138 ±9	85* ±15	per cent

* ≥ 0.05 (not significantly different from the controls).

Table VI. Unscheduled DNA synthesis (UDS) and poly(ADP-ribose)polymerase-activity (PARP) of chicken embryo brain- and liver -cells immediately following *in vitro* – exposure to bleomycin (BM) and X-rays. Control values: UDS brain-cells: $(0.35 \pm 0.07) \times 10^6$ dpm/µg DNA, UDS liver-cells: $(0.23 \pm 0.05) \times 10^6$ dpm/µg DNA, PARP brain-cells: 144±59 pmol ^{14}C-NAD utilized per mg DNA, PARP liver-cells: 181±59 pmol ^{14}C-NAD utilized per mg DNA.

Agent	**Cells**	**UDS**		**PARP***	
		Approximate threshold dose	Maximal effect	Approximate threshold dose	Maximal effect
Bleomycin	brain-	35 nM	+ 100%	35 nM	+ 140%
	liver-	35 nM	+ 60%	35 nM	+ 40%
X-Rays	brain-	8 Gy	+ 20%	5 Gy	+ 30%
	liver-	8 Gy	+ 20%	no effect	no effect

* From previous investigations (Ignatius *et al.*, 1992).

Discussion

Like the cellular effects of ionizing radiation (see, e.g., Nunez *et al.,* 1996; Olive, 1998), the cytotoxicity of BM seems to be highly related to the induction of single- and double-strand breaks in DNA (Burger, 1998; Whisnant-Hurst and Leadon, 1999). As shown by many investigations (see, e.g., Cook and Brazell, 1975; Weniger, 1982; Wang *et al.,* 1997) DNA strand breaks lower the compactness, i.e., DNA supercoiling, and thereby the sedimentation rate of nucleoids (Cook and Brazell, 1975; Weniger, 1982; Singh *et al.,* 1995).

While the reduction in the sedimentation of nucleoids following X-irradiation of the chicken embryo cells *in vitro* within a defined dose-range, i.e. 2–16 Gy (Table II), could be expected, the increase in nucleoid sedimentation at doses of ≥ 16 Gy above the level reached at 8 Gy seems to be surprising. The same is true with regard to the fact that BM, following a slight decrease in the sedimentation distance at concentrations of ≤ 23.6 nM, induces a highly significant and dose-dependent increase in nucleoid sedimentation up to about 200 per cent of the control values at concentrations of about 47 to 377 nM (Table I), suggesting an elevation of nucleoid compactness at very high doses of X-rays and BM. As shown by previous investigations, chicken embryo cells exhibit significant signs of spontaneous and induced apoptotic cell death (Tempel and Ignatius, 1993). Whereas cleavage of DNA by DNases does not appear to be an invariable feature of apoptosis (Ueda and Shah, 1994; Baumgartner-Parzer, 1996; ibid further references), shrinking of apoptotic cells and chromatin condensation are regarded to be rather constant characteristics of this kind of cell death (see, e.g., Kohn *et al.,* 1995; Bayly *et al.,* 1997). DNA strand breaks and DNA fragmentation lower chromatin compactness and, thereby nucleoid sedimentation, whereas an increase in chromatin condensation is accompanied by lengthening of the nucleoid sedimentation distance (Mattern *et al.,* 1987). It is suggested by the results presented in Tables I and II that both phenomena, i.e. DNA strand breaks and chromatin condensation, influence the sedimentation analysis in such a manner that, within the lower dose range DNA strand breaks and at higher doses the increase in chromatin condensation prevail. The same is true with regard to the viscosity of alkaline cell lysates, exhibiting an increase at very low BM doses and lowering within the higher dose-range (Table I). Contrary to BM, an increment of viscosity could not be seen in X-irradiated cells though a diminuation of the nucleoid sedimentation distance being evident at doses of 2–16 Gy (Table II). It is highly probable therefore that in X-irradiated chicken embryo cells apoptotic chromatin condensation occurs at a lower concentration of DNA strand breaks than in cells exposed to BM.

As evident from the results presented in Table III, SDS was increased, *in vitro*, at rather low BM concentrations and diminished, in a dose-dependent manner, within the higher dose-range and after X-irradiation. Exposure of chicken embryos to X-rays *in ovo* (at doses of 4 and 8 Gy) was followed by a decrease in SDS to approximately 30 and 50 per cent in liver- and brain-cells, respectively (Stammberger *et al.,* 1989; Tempel *et al.,* 1992). Investigations in chicken embryo cells following *in ovo* exposure presently were limited to BM. As can be seen from the results presented in Tables IV and V, the outcomes of viscometry, nucleoid sedimentation and SDS assays roughly paralleled the results obtained following the exposure of the cells *in vitro.* An increase in viscosity and dT-^{3}H incorporation and a very slight decrease in nucleoid sedimentation is seen a the lower dose-range (1.45 and 2.90 μg BM/egg) whereas the contrary is evident at BM doses ≥ 29 μg/egg (Table IV). As regards to the BM-induced effects, a dose of 1.45 μg/egg was followed, within 6–12 hours, by an increase in viscosity of cell lysates and in SDS by almost 50–60 per cent (Table V). It could be shown by the present investigations and by previous studies in chicken embryo cells that BM and X-rays exhibit significant differences in two DNA-repair systems, i.e., UDS (e.g., Seldon *et al.,* 1994) and PARP (LeRhun *et al.,* 1998) (Table VI). Regarding the characteristics of "long-patch DNA repair" in BM-damaged DNA (Whisnant-Hurst and Leadon, 1999) as compared to the "short-patch DNA-repair" in X-irradiated cells (Francis *et al.,* 1981), one may argue that the increase in viscosity observed in parallel to the increase in UDS and SDS following exposure to BM *in vitro* and *in ovo* reflects the activation of DNA-repair systems. – With BM-induced SDS stimulation, histological analysis did not re-

veal any signs of increased proliferation or mitotic rate (Schmahl, personal communication).

Conclusions

1.) Chicken embryo cells respond to BM at remarkably low concentrations.
2.) Even rather simple short-term tests are able to reveal significant differences between the "radiomimetic drug" BM and X-rays.
3.) After exposure to BM, chicken embryo cells exhibit a rapid and extensive post-treatment DNA repair which is different from that one seen in X-irradiated cells.
4.) In apoptosis-prone cells, like the brain- and liver-cells of the chicken embryo, viscometry of alkaline cell-lysates and nucleoid sedimentation reflect two opposite phenomena, DNA fragmentation and chromatin condensation.
5.) In the course of further investigations, more sophisticated methods, such as the single cell gel electrophoresis, should be used including other tissues and stages of development.

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