REPAIR OF POTENTIALLY LETHAL DAMAGE AFTER SINGLE INJECTION OR CONTINUOUS INFUSION OF BLEOMYCIN

Yasuo SUZUKI,*2 Muneyasu URANO,*3 Koichi ANDO,*4 Takeshi TODOROKI, and Sachiko KOIKE

Division of Clinical Research, National Institute of Radiological Sciences*1

The repair of potentially lethal damage after administration of bleomycin by a single injection or by continuous infusion was studied in vivo. Experimental tumors were the fifth generation isotransplants of a squamous cell carcinoma which arose spontaneously in a C3Hf/He female mouse. Bleomycin was administered intraperitoneally by a single injection or by 24-hr continuous infusion. Cell survival was assayed by TD50 method. Dose–survival curve after a single injection exhibited a biphasic or upward concave curve. Surviving fraction increased rapidly if tumors were left in situ after treatment and reached a plateau at 5 hr, indicating that tumor cells were able to repair the potentially lethal damage induced by bleomycin. Dose–survival curve after continuous infusion was also biphasic, but had a small shoulder. The repair of potentially lethal damage was slight, if any, when tumors remained in situ for 6 hr after the end of 24-hr continuous infusion. This indicates that potentially lethal damage was being repaired during drug infusion.

Hahn and his colleagues3) examined the response of EMT6 mouse mammary tumor to chemotherapeutic agents and found that survival of the tumor cells increased if tumors were left in situ for 24 hr after treatment. They suggested that the repair of potentially lethal damage most satisfactorily explained this increase of survival. The increase was found most remarkable after bleomycin treatment. Since then several groups studying bleomycin have demonstrated the repair of potentially lethal damage occurring after the drug administration in vitro7,13) and in vivo9,12,13) systems. The repair has also been studied after fractionated treatments. Chinese hamster ovary cells failed to repair after the second bleomycin dose,1) while EMT6 tumor cells were shown to be capable of repairing each potentially lethal damage induced after fractionated doses in vivo.14) C3H mouse squamous cell carcinoma was also found to be capable of repairing the damage given by fractionated radiation.15) The repair capability of this kind in tumor cells is an essential factor for designing tumor treatment, particularly when a multi-dose schedule is employed. In the present work, we studied this point, either after single rapid injection or after continuous infusion of bleomycin in vivo.

MATERIALS AND METHODS

Experimental animals were 8- to 12-week-old C3Hf/He mice which have been maintained in...
our Institute under specific pathogen-free (SPF) conditions. Animals were provided with sterilized Purina pellets and chlorinated water freely. The fifth generation isotransplants of a squamous cell carcinoma which arose spontaneously in a C3Hf/He female mouse were used. The method of obtaining these tumor cells was similar to that described for C3H mouse mammary carcinoma. Briefly, first to third generation tumors were stored in a liquid nitrogen refrigerator and fourth generation tumors were grown in flank tissues of female mice for experimental use.

Single-cell suspension was prepared as follows: Animals with fourth generation isotransplants were sacrificed by cervical dislocation. Tumors were excised and necrotic portions were carefully removed. Intact tumor tissues were minced with scissors and trypsinized at 37°C for 30 min in a flask which contained 0.2% trypsin (Difco, 1:250) in Ca- and Mg-free Dulbecco's solution. The cell suspension was allowed to settle for 15 min in crushed ice. Approximately two-thirds of the supernatant was removed carefully by a syringe, and centrifuged at 1,600 rpm for 5 min. The sediment was resuspended with a sufficient amount of Hanks' medium containing 5% fetal calf serum. Viable tumor cells were counted in a hemocytometer by use of the Trypan Blue staining method. Ten µl of this single-cell suspension (approximately 10⁵ unstained tumor cells) was transplanted into the subcutaneous tissue of the right thigh.

Cell survival was assayed by the TD₅₀ method, in which the number of tumor cells required for tumor growth in one-half the transplanted sites was determined. Tumors were treated with bleomycin when reaching 8~9 mm in diameter and single-cell suspension was prepared. The cell suspension containing a known number of viable tumor cells was serially diluted in 3-fold with Hanks' medium into 6 to 8 doses. Each dilution was injected subcutaneously into both thighs. Recipients were given whole-body irradiation of 450 rad from a ¹³⁷Cs irradiator (dose rate was approximately 100 rad/min) and were randomly arranged in groups 24 hr before transplantation. Tumor "takes" were examined by palpating the transplanted regions every 7 days. Tumors which grew larger than 10 mm in diameter in 60 days after transplantation were scored as "takes" and TD₅₀ was calculated by the logit method. The surviving fraction of drug-treated tumor cells was obtained from the ratio of TD₅₀ of non-treated to TD₅₀ of drug-treated. In a single TD₅₀ assay, approximately 10 male and 10 female mice were employed and 4~6 assays were usually performed simultaneously.

Bleomycin complex (copper free) was kindly supplied by Nippon Kayaku Co., Tokyo. The agent was dissolved in distilled water and administered intraperitoneally by a single rapid injection or by continuous infusion through a catheter (intravenous catheter for cut down; external diameter, 1 mm; Atom Co., Tokyo) for 24 hr using a motor-driven infusion pump at the rate of 1 ml/24 hr.

RESULTS

Animals were treated with a single rapid dose of bleomycin of 7.5 mg/kg and tumors were removed at various periods thereafter. As shown in Fig. 1, survivals increased rapidly if tumors were allowed to remain in situ for more than 2 hr after treatment and almost reached a plateau at 5 hr. Surviving fraction of tumor cells removed immediately after treatment was 0.003, while that of tumor cells removed at 6 hr increased to 0.3, i.e., approximately 100-fold increase of survival was found in the 6-hr interval.

The dose–survival curves of tumor cells treated with graded doses of bleomycin were

Fig. 1. Increase of survival as a function of time remaining in situ after bleomycin administration

Bleomycin (7.5 mg/kg) was administered at time 0. Vertical bars represent 95% confidence limit.
determined at 30 min or 6 hr thereafter. The lower curve in Fig. 2 demonstrates a dose–response curve of tumor cells removed 30 min after treatment while the upper curve shows that of tumor cells excised 6 hr thereafter. The magnitude of the increase in survival fraction was found to depend on bleomycin dose. Both dose–response relations exhibit biphasic or upward concave curves, without a shoulder. The lower curve shows an extensive 30-min survival curve. \( D_0 \), the drug dose to reduce survival by a factor of 1/e on the exponential portion of the survival curve, was 0.185 and 30 mg/kg in the initial sensitive portion and in resistant tail portion, respectively. The inflexion point was found in the region of 1–10 mg/kg.

The next step was to study the effect of continuous infusion of bleomycin on survival. Infusion period was fixed at 24 hr for various doses of the drug. As indicated in Fig. 3 the dose–survival curve after continuous infusion (infusion–survival curve) was also biphasic or upward concave with a small shoulder in its initial portion. However, the survival level was remarkably high compared to that of 30-min survival curve, as shown by the dashed line in Fig. 3. At the 15 mg/kg point, tumor cells treated with 24-hr infusion showed approximately 100 times higher survival than those treated with a single rapid dose. Open circles indicate survival of tumors treated with 24-hr infusion and left in situ for 6 hr thereafter, which was found slightly above the infusion–survival curve while no significant differences were observed.

An experiment was attempted in order to elucidate the small shoulder in the infusion–survival curve. Constant amount of bleomycin (0.75 mg/kg) was given for various infusion periods and animals were sacrificed at the end of each treatment. Surviving fractions are shown as a function of the infusion period in Fig. 4, where the surviving fraction 30

---

**Fig. 2.** Dose–survival curves of tumor cells treated with graded doses of bleomycin
- Survival of tumor cells removed 30 min after rapid injection (30-min survival curve), ○ survival of tumor cells left in situ for 6 hr (6-hr survival curve).

**Fig. 3.** Dose–response curve of tumor cells treated with 24-hr infusion of graded doses of bleomycin
- Survival of tumor cells removed immediately after treatment (infusion–survival curve), ○ survival of tumor cells left in situ for 6 hr after the end of infusion. --- 30-min survival curve superimposed from Fig. 2.
min after the start of infusion was 0.06, increased with time, and attained unity at 12 hr. This result indicated that a low concentration of bleomycin, if given by infusion over a long time, gave no lethal damage to tumor cells.

Fig. 4. Survival of tumor cells as a function of infusion period
Animals received 0.75 mg/kg of bleomycin during various infusion periods and survivals were assayed immediately thereafter.

**DISCUSSION**

Our murine squamous cell carcinoma exhibited, as well as many other cell lines, biphasic or upward concave survival curve after a single dose of bleomycin. Rapid increase of survivals was also observed if tumors were left in situ for several hours after treatment. As suggested by other authors, the most satisfactory explanation for this increase would be that our tumor cells were able to repair the potentially lethal bleomycin damage. Phillips and Tolmach first suggested that HeLa cells cultured in vitro were capable of repairing potentially lethal damage induced by irradiation if these cells were kept under appropriate post-irradiation conditions. Subsequently, the same phenomenon was observed in murine tumors, either in ascites form or in solid form. This kind of repair was also found after chemotherapy. Hahn *et al.* observed this in EMT6 mammary sarcoma treated with three different chemotherapeutic agents (cyclophosphamide, 5-fluorouracil, and bleomycin) and Twentyman showed remarkable survival increase of the same tumor cells after bleomycin treatment. Takabe *et al.* reached a similar conclusion with Ehrlich ascites tumor cells where a possible effect of trypsin, a cell-dispersing agent, was entirely excluded.

There is a strong demand in cancer therapy to develop physical or biological methods which can inhibit the repair of potentially lethal damage induced by irradiation or by drug treatment. Braun and Hahn recently found that 43° hyperthermia prevented the repair of potentially lethal bleomycin damage in cultured Chinese hamster cells, and Barranco *et al.* also reported that Chinese hamster ovary cells treated with nitrosourea compound failed to recover from potentially lethal damage. Takabe *et al.* showed that a continuous bleomycin treatment inhibited the repair of potentially lethal damage in Ehrlich ascites tumor cells and resulted in greater cell killing. Accordingly, we examined the effect of continuous infusion of the antibiotic on solid murine tumor. The dose–survival curve after continuous infusion was also upward concave, but the tumor cells used were less sensitive to this treatment than to a single rapid injection. The survivals did not increase significantly when tumors were left in situ for 6 hr after the end of 24-hr continuous infusion (Fig. 3). However the increase of survivals was noted when a constant amount of bleomycin was infused for various periods, i.e., the increase of survival depended on the infusion period (Fig. 4). These results indicated that tumor cells were repairing potentially lethal damage during the continuous infusion. The infusion–survival curve and the 6-hr survival curve are very alike, except for a small shoulder featuring the former. This suggests that con-
Continuous infusion might be as effective as a single rapid dose if an appropriately higher dose is given, while it might be less effective than a single rapid dose if a smaller total dose is infused. It must be pointed out that continuous infusion was made over a 24-hr period. This might have affected the progression of tumor cells through the cell cycle for 24 hr, and resulted in an additional effect on tumor growth. Previous studies in our laboratory demonstrated that 4-day infusion of bleomycin inhibited the growth of spontaneous C3H mouse mammary carcinoma more effectively than fractionated injections of the same total dose. Accordingly, we might conclude that continuous infusion inhibits the tumor growth by slowing cell progression and results in an additional effect, while its dose-response relationship is similar to the 6-hr survival curve after single doses and repair of potentially lethal damage takes place during the infusion period.

(Received October 6, 1977)

References

12) Twentyman, P. R., Bleehen, N. M., Br. J. Cancer, 30, 469−472 (1974).