DeLoach J.R., Peters S., Pinkard O., Glew R. and Ihler G. (1977) Effect of glutaraldehyde treatment on enzyme-loaded erythrocytes. *Biochim. Biophys. Acta* 496: 507-515.

DeLoach J.R., Tagner C.H. and Barton C. (1983) Hepatic pharmacokinetics of glutaraldehyde-treated methotrexate-loaded carrier erythrocytes in dogs. *Res. Exp. Med.* 183: 167-175.

Gasparini A., Tonetti M., Astroff B., Rowe L., Satterfield W., Schimdt R. and DeLoach J.R. (1992) Pharmacokinetics of doxorubicin loaded and glutaraldhyde treated erythrocytes in healthy and lymphoma bearing dogs. In: *Use of Resealed Erythrocytes as Carriers and Bioreactors* (Magnani M. and DeLoach J.R., eds.), pp. 299-304, Plenum Press, New York.

Gaudreault R.C., Bellemore B and Lacroix J. (1989) Erythrocyte membrane-bound daunorubicin as a delivery system in anticancer treatment. *Anticancer Res.* 9: 1201-1206.

Magnani M., Rossi L., Brandi G., Schiavano G., Montroni M. and Piedimonte G. (1992) Targeting of antiretroviral nucleoside analogous in phosphorylated form to macrophages: in vitro and in vivo studies. *Proc. Natl. Acad. Sci. USA* 89: 6477-6481.

Tonetti M., Astroff B., Satterfield W., De Flora A., Benatti U. and DeLoach J.R. (1990) Construction and characterization of adriamycin -loaded canine red blood cells as a potential slow delivery system. *Biotechnol. Appl. Biochem.* 12: 621-629.

Tonetti M., Astroff B., Satterfield W., De Flora A., Benatti U. and DeLoach J.R. (1991a) Pharmacokinetic properties of doxorubicin encapsualted in glutaraldehyde-treated canine erythrocytes. *Am. J. Vet. Res.* 52: 1630-1635.

Tonetti M., Zocchi E., Guida L., Polvani C., Benatti U., Biassoni P., Romei F., Guglielmi A., Aschele A., Sobrero A. and De Flora A. (1991b) Liver targeting of autologous erythrocytes loaded with doxorubicin. *Eur. J. Cancer* 27: 947-948.

Tonetti M., Zocchi E., Guida L., Polvani C., Benatti U., Biassoni P., Romei F., Guglielmi A., Aschele A., Sobrero A. and De Flora A. (1992) In: *Use of Resealed Erythrocytes as Carriers and Bioreactors* (Magnani M. and DeLoach J.R., eds.), pp. 307-317, Plenum Press, New York.

Zocchi E., Guida L., Benatti U., Canepa M., Borgiani L., Zanin T. and De Flora A. (1987) Hepatic or splenic targeting of carrier erythrocytes: a murine model. *Biotechnol. Appl. Biochem.* 9: 423-434.

Zocchi E., Tonetti M., Polvani C., Guida L., Benatti U. and De Flora A. (1988) In vivo liver and lung targeting of adriamycin encapsulated in glutaraldehyde-treated murine erythrocytes. *Biotechnol. Appl. Biochem.* 10: 555-562.

Zocchi E., Tonetti M., Polvani C., Guida L., Benatti U. and De Flora A. (1989) Encapsulation of doxorubicin in liver-targeted erythrocytes increases the therapeutic index of the drug in a murine metastatic model. *Proc. Natl. Acad. Sci. USA* 86: 2040-2044.

Pergamon

Advances in the Biosciences, Vol. 92, pp. 177–183, 1994

0065-3446/94 $26.00

Treatment of Rausher Virus Induced Murine Erythroblastic Leukemia with Rubomycin Loaded Erythrocytes

F.I. Ataullakhanov*, T.V. Batasheva*, V.M. Bukhman**, S.V. Komarova*, T.D. Oreshkina** and V.M. Vitvitsky*

*Scientific Center for Hematology of RAMS, 125167, Moscow, Russia
**Institute of New Antibiotics of RAMS, 119867, Moscow, Russia

ABSTRACT

BALB/c female mice with advanced erythroblastic leukemia received two i.v. injections of rubomycin with a 4 day interval. Rubomycin was injected either as the drug solution or as a suspension of rubomycin loaded erythrocytes. During each injection mice received 0.2 ml of the preparation containing 10, 20 or 30 mg of rubomycin per kg of body weight. The first rubomycin injection was made when average spleen weight was 2-9 times greater than normal one. On the next day after the second rubomycin injection mice were killed. The antileukemic effect of the preparation was evaluated as an inhibition of splenomegaly development and toxic effect was characterized by body weight loss. Untreated mice had spleen weight 6-23 times greater the normal one. In all cases rubomycin treatment provided strong antileukemic effect. All mice which were receiving rubomycin as rubomycin loaded erythrocytes and those which were receiving 10 mg/kg of rubomycin as drug solution had spleen weights near the normal value. Spleen weights of mice treated with drug solution in doses of 20 or 30 mg/kg were significantly below the normal value (up to 30% of the normal weight in the last case). Toxic effect of rubomycin loaded erythrocytes was significantly weaker than that of the drug solution. Treatment of rubomycin loaded erythrocytes with glutaraldehyde did not alter their therapeutic efficacy. Moreover, such glutaraldehyde treated erythrocytes retained their therapeutic efficacy after freezing in liquid nitrogen.

KEYWORDS

Carrier erythrocyte; leukemia; mouse; rubomycin; treatment

INTRODUCTION

Rubomycin (daunorubicin), anthracycline antibiotic, is an effective antileukemic drug, but its administration does not permit the complete remission to be achieved in all cases and may be accompanied with severe side effects related to its high toxicity (Cassiletti and Katz, 1977; Stenberg *et al.*, 1991). Numerous attempts have been made to improve the therapeutic index of anthracycline antibiotics by using different drug carriers (Ataullakhanov *et al.*, 1992; Kitao *et al.*, 1978; Kitao and Hattori, 1980; Oreshkina *et al.*, 1993; Rachman *et al.*, 1986; Tonetti *et al.*, 1990; Willmot *et al.*, 1985; Zocchi *et al.*, 1988, 1989). The possibility of the therapeutic efficacy improvement of adriamycin (doxorubicin), the other antracycline antibiotic, by using of drug carrier erythrocytes was demonstrated using murine models (Zocchi *et al.*, 1988, 1989). Enhancement efficacy of rubomycin loaded erythrocytes (RLE) if compared with drug solution, was found in treatment of murine ascite tumor L1210 (Kitao *et al.*, 1978; Kitao and Hattori, 1980). Recently it was found that human and murine erythrocytes might be loaded with rubomycin by simple incubation of erythrocytes in rubomycin containing isotonic solution and therapeutic efficacy of such RLE similar to that of drug solution was demonstrated in the other murine ascite tumor P388 (Ataullakhanov *et al.*, 1992). Choice of suitable model may be very important for evaluation of the therapeutic efficacy of drug carrier erythrocytes. Moreover, the comparison of their effect using different models is very desirable. In this work we compared the effects of intravenously injected RLE and rubomycin solution using as a model Rausher virus induced murine erythroblastic leukemia.

MATERIALS AND METHODS

Murine Leukemia Induction. Rauscher virus containing BALB/c mice serum was stored at -196°C. Before use the serum was thawed and diluted in 199 Medium up to titer of virus near 10^4 spleen CFU/ml (Pluznik and Sachs, 1964). For leukemia induction 0.2 ml of diluted virus containing serum was injected intravenously to female BALB/c mice of 18-20 g body weight. Leukemia progress was monitored by splenomegaly development.

Preparation of Rubomycin Loaded Erythrocytes (RLE). Human or mice blood was collected with standard glucose-citrate preservative solution. Erythrocytes were separated by centrifugation and washed twice in double volume of glucose containing phosphate buffered saline (GPBS). Washed erythrocytes were mixed with double volume of rubomycin solution in GPBS. Pharmaceutic preparation of rubomycin produced by Mosmedpreparaty, USSR was used. The mixture was incubated at room temperature for 40 min resulting in binding of near 80% of rubomycin presented in the medium to erythrocytes (Ataullakhanov *et al.*, 1992, 1993). Erythrocyte bound of rubomycin was evaluated by measuring rubomycin concentration in the incubation medium (Ataullakhanov *et al.*, 1993). Erythrocytes with different rubomycin content were prepared using different rubomycin concentration in the incubation medium. Final rubomycin concentration in erythrocytes was calculated using data on rubomycin concentration decrease in the incubation medium and hematocrit value of the suspension. After it RLE were sedimented by centrifugation and either suspended in GPBS up to hematocrit value of 50% and used for injection or treated with glutaraldehyde. Glutaraldehyde treatment of RLE was performed by their mixing with an equal volume of glutaraldehyde solution in GPBS followed by incubation for 40 min at room temperature and cell washing as it was described by Tonetti *et al.*, (1990). Glutaraldehyde treated RLE were used also as 50% suspension in GPBS. RLE with or without glutaraldehyde treatment were prepared just before use.

Treatment of Rauscher Virus Induced Leukemia. Rauscher virus induced leukemia treatment was started when a pronounced splenomegaly developed. Spleen size was determined by palpation and by control killing of some mice for spleen weighing. Leukemic mice received two intravenous injections of 0.2 ml rubomycin preparation at 4 day interval. Rubomycin was injected either as the drug solution or RLE suspension. The first rubomycin injection was made when average spleen weight was 2-9 times greater than normal one. On the next day after the second rubomycin injection mice were killed and the effect of treatment was evaluated by alterations in spleen and body weights. The antileukemic effect of the preparations was evaluated by inhibition of splenomegaly development and toxic effect was evaluated by body weight loss.

RESULTS

In the preliminary experiment the efficacy of different rubomycin doses in treatment of Rausher virus induced leukemia were tested. During this experiment rubomycin was administrated either as molecular drug solution or as human RLE. The results are summarized in Table 1. As one can see, in all cases antileukemic effect consisting in the termination of splenomegaly development and decrease of spleen weight was achieved. Simultaneously a body weight loss was observed indicating toxic effect of the preparations. However, when equal doses of rubomycin were used for injection the magnitude of these effects depended strongly on the type of preparation. Spleen weight was lower and body weight loss was significantly greater after administration of rubomycin solution if compared with those, observed when the same rubomycin dose was administered inside human erythrocytes. Moreover, when rubomycin solution was injected in a dose of 30 mg/kg, dramatic decrease of spleen weight (up to one third of the normal one) was observed demonstrating the prevalence of the toxic effect over the antileukemic one. Drug related death of one animal during the experiment occurred in this group. At the same time all mice received suspension of rubomycin loaded erythrocytes had spleen weight not far from normal value. Rubomycin dose of 20 md/kg of body weight was chosen for further investigation because it supported the normal spleen weight after treatment with RLE and provided satisfactory results after administration of rubomycin solution.

Table 1. Comparison of antileukemic and toxic effects of different rubomycin doses injected as drug solution or as suspension of human RLE on mice bearing Rauscher virus induced leukemia. First drug injection was made on the day 9 after virus injection.

Drug	Dose per injection (mg/kg bw)	Spleen weight (mg)	Fraction of normal	Body weight change (g)	Drug related death
Solution	10	111 ± 15	0.8	-4.2 ± 1.0	0/8
-	20	87 ± 9	0.6	-6.4 ± 0.6	0/8
-	30	48 ± 5	0.3	-6.8 ± 0.4	1/8
Human RLE	10	194 ± 14	1.3	-1.4 ± 0.4	0/8
-	20	154 ± 14	1	-3.6 ± 0.4	0/8
-	30	111 ± 9	0.8	-3.9 ± 0.4	0/8
Before treatment		342 ± 80	2.3	-	0/4
Untreated		877 ± 47	6.0	+3.0 ± 0.4	0/8
Control		146 ± 8	1.0	+0.6 ± 0.2	0/8

Using this dose in murine RLE we had obtained results (Table 2) similar to those obtained with human RLE. In these experiments glutaraldehyde treated murine RLE were also used. Preliminary it has been shown that glutaraldehyde concentration of 0.35 % is optimal for erythrocyte treatment. Such treatment was investigated keeping in mind the necessity of RLE preservation. Without glutaraldehyde treatment RLE demonstrate an intensive rubomycin and hemoglobin loss (Ataullakhanov *et al.*, 1992, 1993) and are destroyed within few hours. RLE treated with 0.1 % glutaraldehyde solution were found to have poor stability. They showed significant rubomycin and hemoglobin leakage and low resistance towards freezing and thawing. On the other hand RLE treated with 1% glutaraldehyde solution were fairly stable but they formed multiple aggregates resulting in animal death after i.v. injection. RLE treated with 0.35% glutaraldehyde solution were shown to have negligible rubomycin and hemoglobin outflux. They formed very few aggregates and recovered well after freezing and thawing procedure. Antileukemic activity and toxicity of RLE treated with 0.35% glutaraldehyde solution were similar to those of untreated RLE (Table 2). Moreover, they retain the therapeutic effect after freezing in liquid nitrogen and thawing (Table 3). It suggests that storage of this preparation is feasible.

Table 2. Antileukemic and toxic effects of rubomycin injected either as drug solution or as suspension of murine RLE or as suspension of glutaraldehyde treated murine RLE on mice bearing Rauscher virus induced leukemia. First drug injection was made on the day 8 after virus injection in experiment #1 and on the day 10 in experiment #2.

Drug	Dose per injection (mg/kg bw)	Spleen weight (mg)	Fraction of normal	Body weight change (g)	Drug related death
		Experiment #1			
Solution	20	120 ± 30	0.6	-6.4 ± 0.6	2/6
Murine RLE	-	131 ± 11	0.7	-4.7 ± 0.8	0/6
GT murine RLE	-	173 ± 32	0.9	-4.3 ± 0.7	0/6
Before treatment		900	4.8	-	0/2
Untreated		1518 ± 153	8.1	+0.9 ± 0.4	0/8
Control		186 ± 17	1.0	-1.5 ± 0.5	0/7
		Experiment #2			
Solution	20	102 ± 10	0.7	-4.1 ± 0.6	1/7
Murine RLE	-	216 ± 53	1.4	-2.9 ±0.6	1/7
GT murine RLE	-	233 ± 12	1.5	-2.2 ± 0.4	0/7
Before treatment		811 ± 45	5.3	-	0/7
Untreated		1330 ± 43	8.7	+0.2 ± 0.1	0/7
Control		153 ± 11	1.0	+1.1 ± 0.6	0/9

Table 3. Comparison of antileukemic and toxic effects of glutaraldehyde treated murine RLE before and after freezing in liquid nitrogen. First drug injection was made on day 12 after virus injection.

Drug	Dose per injection (mg/kg bw)	Spleen weight (mg)	Fraction of normal	Body weight change (g)	Drug related death
Solution	20	233 ± 21	1.8	-5.9 ± 0.4	0/10
GT RLE	-	452 ± 91	3.5	-3.7 ± 0.6	0/ 9
GT RLE after freezing	-	362 ± 46	2.8	-3.4 ± 0.4	0/10
Before treatment		1200 ± 155	9.2	-	0/ 6
Untreated		3032 ± 224	23.1	-4.1 ± 0.6	0/14
Control		131 ± 11	1.0	+0.7 ± 0.01	0/ 7

DISCUSSION

Rausher virus induced murine leukemia seems to be more suitable model for the evaluation of RLE therapeutic efficacy than earlier used murine ascite tumors (Ataullakhanov *et al.*, 1992; Kitao *et al.*, 1978; Kitao and Hattori, 1980). This model assumes the intravenous drug injection which is rather usual mode of rubomycin administration. It also permits the antitumor and toxic effects of the drug preparations to be distinguish. The strong antileukemic effect of RLE was obtained in our experiments. However the antileukemic activity of RLE was lower than that of rubomycin solution. This decrease of activity hardly can be explained by degradation of rubomycin entrapped into erythrocytes, because the equal or increased antitumor activity of RLE in comparison with the rubomycin in solution was found for other murine models (Ataullakhanov *et al.*, 1992; Kitao *et al.*, 1978; Kitao and Hattori, 1980). So it may be assumed that the ratio of RLE and rubomycin solution antitumor activities is specifically determined by testing model used. Significantly lower toxic effect (evaluated by body weight loss as well as by drug related death) of RLE in comparison with that of drug solution was obtained in this work . We assume that the decreased toxicity of RLE is not related to the testing model and demonstrates the advantage of this formulation. No difference in therapeutic effects of glutaraldehyde treated RLE and untreated ones was found. However, the glutaraldehyde treated RLE may be more suitable formulation because they are more stable and allow the preparation and storage rather long before usage for treatment.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. R.I. Volkova and Dr. S.M. Kulikov for help in the preparation of the manuscript.

REFERENCES

Ataullakhanov, F.I., Vitvitsky, V.M., Kovaleva, V.L. and S.B. Mironova (1992). Rubomycin loaded erythrocytes in the treatment of mouse tumor P388. In: *Advances in Experimental Medicine and Biology* (M. Magnani and J.R. DeLoach, eds.), Vol. 326, pp. 209-213. Plenum Press, New York.

Ataullakhanov, F.I., Batasheva, T.V., Vitvitsky, V.M. and S.V. Komarova (1993). Effect of glutaraldehyde treatment on rubomycin and hemoglobin leakage from murine erythrocytes loaded with rubomycin. *Biotekhnologiya*, 2, 40-43.

Cassiletti, P.A. and M.E. Katz (1977). Chemotherapy for adult acute non lymphocytic leukemia with daunorubicin and cytosine arabinoside. *Cancer Treat. Rep.*, 61, 1441-1445.

Kitao, T., Hattori, K. and M. Takeshita (1978). Agglutination of leukemic cells and daunomycin entrapped erythrocytes with lectin *in vitro* and *in vivo*. *Experientia*, 34, 94-95.

Kitao, T. and K.Hattori (1980). Erythrocyte entrapment of daunomycin by amphotericin B without hemolysis. *Cancer research*, 40, 1351-1353.

Oreshkina, T.D., Korolev, A.M., Preobrazhenskaya, M.N. and V.M. Bukhman (1993). Selective antitumor effect of amid of daunorubicin and N-carboxymethylascorbigen hydrosol against murine advanced erythroblastic leukemia (MAEL). *18th International Congress of Chemotherapy*, June 27-July 2, 1993, Stockholm, Sweden, Abstracts 1169, p. 317.

Pluznik, D.H. and Z. Sachs (1964). Quantitation of a murine leukemia virus with spleen colony assay. *J. Nat. Cancer Inst.*, 33, 535-546.

Rachman, A., Fumagalli, A., Barbieri, B., Schein, P. and A.M. Casazza (1986). Antitumor and toxicity evaluation of free dixorubicin and dixorubicin entrapped in cardiolipin liposomes. *Cancer Chemother. Pharmacol.*, 16, 22-27.

Stenberg, C.P., Civin, C., Krischer, J., Culbert, S., Ragab, A., Ruymann, F.B., Ravindranath, J., Leventhal, B., Wilkinson, R. and T.J. Vietti (1991). A Comparison of induction and maintenance therapy for acute nonlymphocytic leukemia in childhood: results of a pediatric oncology group study. *J. Clin. Oncol.*, 9, 247-288.

Tonetti, M., Astroff, B., Satterfield, W., DeFlora, A., Benatti, U. and J.R. DeLoach (1990). Construction and characterization of adriamycin-loaded canine red blood cells as a potential slow delivery system. *Biotechnol. Appl. Biochem.*, 12, 621-629.

Willmott, N., Cumminds, J., Stuart, J.F.B. and A.T. Florence (1985). Adriamycin-loaded albumin microspheres: preparation, in vivo distribution and release in the rat. *Biopharm. Drug Dispos.*, 6, 91-104.

Zocchi, E., Tonetti, M., Polvani, C., Guida, Z., Benatti, U. and A. DeFlora (1988). *In vivo* liver and lung targeting of adriamycin encapsulated in glutaraldehyde-treated murine erythrocytes. *Biotechnol. Appl. Biochem.*, 10, 555-562.

Zocchi, E., Tonetti, M., Polvani, C.,Guida, Z. Benatti, U. and A. DeFlora (1989). Encapsulation of doxorubicin in liver-targeted erythrocytes increases the therapeutic index of the drug in murine metastatic model. *Proc. Natl. Acad. Sci.USA.*, 86, 2040-2044