Note

A Method for the Induction of Tumor-resistant Mice by Immunization with Diethyl Pyrocarbonate-treated Tumor Cells

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We have reported that incubation with 0.1 M acetate buffer, pH 5.0, at 30°C for 5 min caused tumor cells (MH134) to lose their proliferating activity without loss of their antigenicity.¹⁾ Such attenuated cells induced only 20~25% resistance to a challenge with live MH 134 in C3H mice.²⁾ On the other hand, when these acetate buffer attenuated tumor cells were treated further with diethyl pyrocarbonate (DEPC), 100% of the mice survived the challenge.²⁾ This suggests that modification with DEPC enriched the cellular antigenicity of the transplantable tumors and mice were induced with an immune resistance to the tumor. However, induction of immune resistance by the treatment with DEPC alone was difficult, because the optimum DEPC concentration was different between attenuation of tumor cells and enrichment of cellular antigenicity. Therefore, to obtain an effective immune resistance, an attenuation procedure with acetate buffer was combined, and we have tested whether this procedure of attenuation with acetate buffer, followed by modification with DEPC, can be widely applied for the induction of tumor resistance. Two tumor cell lines, mouse MM2 mammary and mouse L1210 leukemia, were selected for this purpose in the hope that, if the induction of tumor-immunity proves effective in these types of tumors, clinical applications of immuno-therapy might be expected. MM2 is known to carry tumor-associated transplantation antigens, but lacks H-2 antigen.³⁾ L1210 is widely used as a suitable tumor for preclinical screening of chemotherapeutic agents for human leukemia. The induction of tumor resistance to this leukemia is difficult, since L1210 leukemia is highly malignant, grows rapidly, and has low antigenicity.⁴⁻⁶⁾

C3H/He, DBA/2, and C3H mice were purchased from Charles River Japan, Inc. The mouse MM2 mammary cells and L1210 leukemia cells used in this study were

kindly supplied by the Yositomi Pharmaceutical Co., and were maintained by serial transplantation in syngenic C3H/He and DBA/2 mice, respectively. MH134 tumor was kindly supplied by the late Dr. M. Kitagawa and was maintained. DEPC was a gift from Takada Pharmaceutical Industries, Ltd. and was freshly dissolved for each use in Krebs Ringer phosphate buffered solution, pH 7.2, (abbreviated as K-R soln). Ascitic fluids were harvested from C3H/He mice on the 10th day after intraperitoneal inoculation with 10^6 MM2 cells or from DBA/2 mice on the 7th day after intraperitoneal inoculation with 10^6 L1210 cells. MM2 cells and L1210 cells were collected by centrifugation and washed with 0.9% NaCl solution three times by centrifugation at $1,000 \times g$ for 3 min. Tumor cells were attenuated by acetate buffer by the method described previously¹⁾ with a slight modification. Briefly, the tumor cells were suspended in 0.1 M acetate buffer, pH 5.0, containing 0.25 M sucrose at a concentration of 3×10^7 cells/ml and incubated at 30°C for 10 min with gentle shaking. The cells were collected by centrifugation at $1,000 \times g$ for 3 min (designated as AcOH-MM2 or AcOH-L1210). The treatment with DEPC was done by suspending AcOH-MM2 or AcOH-L1210 in 1 ml of K-R soln containing 50 μ g to 500 μ g DEPC and incubating at 30°C for 5 min with gentle stirring. After the reaction, the suspensions were chilled in an ice bath. The cells were collected by centrifugation at $1,000 \times g$ for 3 min and washed twice with cold 0.9% NaCl solution (designated as DEPC-treated AcOH-MM2 or DEPC-treated AcOH-L1210). C3H/He and DBA/2 mice were immunized intraperitoneally with AcOH-MM2 or DEPC treated AcOH-MM2 (3×10^6 cells/mouse) and AcOH-L1210 or DEPC treated AcOH-L1210 (3×10^6 cells/mouse), respectively. Immunization was repeated four times as described in a previous paper.²⁾ Freund's complete adjuvant was added only in the first immunization. Ten days after the fourth immunization, the mice were challenged with live MM2 cells (1×10^5 cells/mouse) or L1210 cells (1×10^3 cells/mouse) intraperitoneally. The survivors without tumors received a further challenge of 1×10^5 MM2 or 1×10^3 L1210 cells on day 30, and the mice which completely rejected the second challenge were regarded as tumor resistant. In these experiments, 6 mice were tested in each group. The experiments were repeated twice.

Figure 1 shows the effects of DEPC treated AcOH-MM2 on the induction of resistance against MM2. A complete resistance to live MM2 cell challenge could be induced in C3H/He mice by the intraperitoneal immunization of 100 μ g and 300 μ g DEPC treated cells. Immunization with AcOH-MM2 did not show such an effect. Figure 2 shows the effects of DEPC treated AcOH-L1210 on the induction of resistance against L1210. Immunization with DEPC treated AcOH-L1210 induced a resistance to the live L1210 cell challenge in DBA/2 mice, but the concentration of DEPC required to induce complete resistance was lower than that of MM2. This low concentration is thought to be due to the smaller size of

Fig. 1. Survival Days of C3H/He Mice Challenged with MM2 Mammary cells after Immunization with AcOH-MM2 or DEPC-treated AcOH-MM2 Cells.

C3H/He mice were immunized four times with AcOH-MM2 or DEPC-treated AcOH-MM2 cells. Ten days after the final immunization, mice were challenged with 10^5 live MM2. —●—, 0.01% DEPC-treated AcOH-MM2; —△—, 0.03% DEPC-treated AcOH-MM2; —□—, 0.05% DEPC-treated AcOH-MM2; —▲—, AcOH-MM2; —■—, unimmunized mice.

Fig. 2. Survival Days of DBA/2 Mice Challenged with L1210 Leukemia after Immunization with AcOH-L1210 or DEPC-treated AcOH-L1210 Cells.

DBA/2 mice were immunized four times with AcOH-L1210 or DEPC-treated AcOH-L1210 cells. Ten days after the final immunization, mice were challenged with 10^3 live L1210. —○—, 0.005% DEPC-treated AcOH-L1210; —●—, 0.01% DEPC-treated AcOH-L1210; —△—, 0.03% DEPC-treated AcOH-L1210; —□—, 0.05% DEPC-treated AcOH-L1210; —▲—, AcOH-L1210; —■—, unimmunized mice.

L1210 cells, because optimum treatment to induce complete anti-tumor resistance for Ehrlich cells and MH134 cells also required 200 μ g to 300 μ g of DEPC per 3×10^7 cells.^{2,7)} Figure 3 shows the protective effect of DEPC against extraction of antigenic materials by 3M KCl. Immunogens were prepared as described in the legend in the figure. Immunization of C3H mice and challenge inoculation were done as in our previous paper.²⁾ Strong resistance was induced in mice when MH134 cells were first treated with DEPC, followed by extraction with 3M

KCl (DEPC treated-3M KCl). Contrary to this, weak resistance was induced in mice when tumor cells were first extracted with 3M KCl, followed by treated with DEPC (3M KCl-DEPC). No resistance was induced when mice were immunized with the cells extracted previously with 3M KCl. These results shows that DEPC protects the antigenic materials from extraction with 3M KCl.

DEPC was first synthesized as a microbicide and kills microbes at a low temperature in a short time.⁸⁾ Wolf suggested that the general feature of the DEPC reaction is

Fig. 3. Survival Days of C3H Mice Challenged with MH134 Hepatoma after Immunization with MH134 Cells Treated Variously with 3M KCl and DEPC.

DEPC-3M KCl was prepared as follows. MH134 cells were first treated with 300 μ g of DEPC dissolved in K-R soln, then cells were incubated with phosphate-buffered saline, pH 7.2, containing 3M KCl at 4°C for 18 hr with gentle stirring. 3M KCl-DEPC was prepared as follows. MH134 cells were first incubated with phosphate-buffered saline, pH 7.2 containing 3M KCl at 4°C for 18 hr with gentle stirring, then cells were treated with 300 μ g of DEPC dissolved in K-R soln.

3M KCl preparation was prepared by incubating MH134 cells in the phosphate-buffered saline containing 3M KCl at 4°C for 18 hr with gentle stirring, then the cell pellet obtained by centrifugation was used as the immunogen. AcOH-DEPC was prepared as in our previous paper.²⁾

C3H mice were immunized four times with the above-described immunogens. Ten days after the final immunization, mice were challenged with 10^5 live MH134. —△—, AcOH-DEPC; —▲—, DEPC-3M KCl; —○—, 3M KCl-DEPC; —●—, 3M KCl; —■—, unimmunized mice.

a formation of intermolecular peptide-like bonds involving amino and carboxylic groups which yield bizarre polymeric proteins.⁹⁾ One possible mechanism of the DEPC reaction could therefore be that a cross-linking reaction between antigen proteins takes place on the cell surface and this antigen cross-linking by DEPC might cause enrichment of the cellular antigenicity. The detailed reaction mechanism of DEPC on the tumor cell surface is under investigation. Anyway, we could induce an immune resistance to MM2 mammary cells in syngenic C3H/He and L1210 leukemia in syngenic DBA/2 by immunizing with DEPC treated AcOH-tumor. This method seems to be a fairly potent immunization procedure for the induction of tumor resistant mice against various tumor cell lines.

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