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Accelerated Regeneration of Trypsin-treated Surface Antigens of Simian Virus 40-transformed BALB/3T3 Cells Induced by X-irradiation[1]

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SUMMARY

The antigens of SV40-transformed BALB/3T3 cells measured by a radioisotopic footpad assay after removal by trypsin treatment regenerated *in vitro* in 3 to 6 hr. After X-irradiation with 3000 R, however, the antigens were regenerated to normal levels within 1 h. X-ray doses of between 1000 and 5000 R accelerated the regeneration of cell surface antigens, while X-irradiation with the larger dose of 8000 R did not. X-irradiation of nontrypsinized tumor cells was without effect. Possible mechanisms of this phenomenon are discussed.

INTRODUCTION

It has been well documented that trypsin treatment produces alterations of cell surface components (2, 8, 11), including decreased antigenicity of tumor cells (5, 6).

We have studied the immunological nature of the cellular immune response against SV40-transformed BALB 3T3 tumor cells inoculated in the footpad of syngeneic or allogeneic mice by a radioisotopic FPA[3] (7, 10). During the course of these experiments, we found that, following removal of the cell surface antigens of these cells with trypsin, the antigens regenerated much more rapidly if the trypsinized cells were X-irradiated. The details of these findings are presented below.

MATERIALS AND METHODS

Animals and Tumors. Inbred strains of male and female BALB/c AnN mice, aged 8 to 12 weeks, were obtained from the NIH Animal Production Section. The E_4 cell line of syngeneic *in vitro* cultured tumor cells was started from a solid fibrosarcoma that developed in a BALB/c mouse inoculated with SV40-transformed BALB/3T3 cells. Cells were grown in Dulbecco-Vogt modified Eagle's minimum essential medium supplemented with 10% fetal calf serum and 1.0% antibiotics solution (5000 units penicillin and 5000 μg streptomycin/ml; Grand Island Biological Co., Grand Island, N. Y.). Cells were grown in roller bottles at 37° and harvested 5 days after subculturing. Nontrypsinized cells were harvested by scraping them with a sterile rubber policeman or shaking vigorously and transferred into sterile screw-cap tubes, which were then centrifuged at 800 × *g* for 5 min and washed 3 times with Hanks' solution. The cells were also harvested by treatment with trypsin (Grand Island Biological Co.). Twenty-five ml of 0.5% trypsin, already at 37°, were added to the cultures, which were then incubated at 37° for 5 min. Medium containing fetal calf serum was added to inactivate the trypsin, and the cells were collected and washed as described above.

Immunization of Mice with Tumor. E_4 immune mice were routinely produced by the s.c. injection of X-irradiated (4000 R) E_4 cells (10^6 cells/mouse) followed by 1 to 3 booster s.c. injections with 1 x 1 x 3-mm pieces of solid E_4 tumor at 7-day intervals. If no growth occurred within 2 weeks after the last challenge, the animals were considered tumor immune and were used in the experiments.

X-irradiation. Nontrypsinized and trypsinized cells in 15-ml plastic tubes (Falcon Plastics, Oxnard, Calif.) were X-irradiated with 1000 to 8000 R using a Westinghouse Quadrocondex X-ray unit, 200 kV, 15 ma, at 25 cm distance from X-ray source.

Radioisotopic FPA. This assay has been described in previous reports (7, 8). Briefly, E_4-immune and normal BALB/c mice are given inoculations of 1×10^6 E_4 cells in the left rear footpad. Immediately thereafter, ^{125}I-labeled syngeneic mouse serum protein (specific activity, 6 to 10×10^6 cpm/mg) is injected i.p. into the mice (10^6 cpm/mouse). Radioiodinated human serum albumin (Mallinckrodt Chemical Works, St. Louis, Mo.) can also be used. Twenty-four or 48 hr later, the test foot and contralateral control foot are cut off at the junction of the lower and middle thirds of the tibia and counted in a gamma spectrometer. Results are expressed as the foot-count ratio: cpm in the test foot divided by cpm in the contralateral control foot. The mean foot-count ratio and S.E. were calculated in experimental and control groups (10 mice/group), and the Student *t* test was used to determine statistical significance of the data.

Treatment with Cycloheximide. Cycloheximide was purchased from Sigma Chemical Co., St. Louis, Mo. E_4 cells were incubated with cycloheximide in a final concentration of 100 μg/ml, which was enough to completely inhibit protein synthesis (S. J. O'Brien, personal communication). Two hr after addition of cycloheximide, the cells were harvested

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[3] The abbreviation used is: FPA, footpad assay.

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by shaking, transferred into sterile tubes, washed 3 times with Hanks' solution, and resuspended in Dulbecco-Vogt modified Eagle's minimum essential medium plus 10% fetal calf serum. Before a FPA, the cells were tested for viability by trypan blue exclusion. There were no detectable effects on cell viability 2 hr after exposure of cells to cycloheximide.

RESULTS

Footpad Reaction Induced by Trypsinized *versus* Nontrypsinized E_4 Cells in Immune and Normal BALB/c Mice. Groups of immune or normal mice were given inoculations in the footpad of washed preparations of trypsin-treated (trypsinized) or scraped (nontrypsinized) cells (1 x 10^6 cells/mouse), and the cellular immune response was measured by the FPA. The experiments were performed with animals that had been immunized with X-irradiated E_4 cells followed by 1 to 3 weekly s.c. injections of live E_4 cells. The results are shown in Table 1. In immune mice that had received 1 booster inoculation of E_4 tumor cells (Group I), the trypsinized E_4 cells gave a reduced foot-count ratio of 1.58 ± 0.03 compared with 2.04 ± 0.07 for the nontrypsinized E_4 cells. In immune mice that had recieved 2 and 3 booster inoculations, trypsinized E_4 cells also gave reduced footpad reactions as opposed to nontrypsinized cells (Groups II and III).

Accelerating Effect of X-irradiation on the Regeneration Time of Cell Surface Antigens Removed with Trypsin. Table 2 shows that the cell surface antigens detected by the FPA have regenerated almost completely between 3 and 6 hr after trypsinization. No regeneration at all occurred in less than 3 hr. By contrast, E_4 cells that had been X-irradiated with 3000 R during the 1-hr period between trypsinization and footpad inoculation induced a reaction equal to that of the nontrypsinized cells.

In order to confirm that regeneration of the cell surface antigens occurred during the period of *in vitro* incubation and not during the 24-hr period *in vivo* after footpad inoculation, the antigenicity of irradiated or nonirradiated trypsinized tumor cells was measured by the FPA after treatment with cycloheximide (Table 3). E_4 cells that had been treated with cycloheximide immediately after trypsinization and irradiation failed to produce a footpad reaction. By contrast, irradiated trypsinized E_4 cells that had been incubated *in vitro* for 1 hr before addition of cycloheximide induced a significant foot-count ratio of 2.05 ± 0.08 com-

Table 1

Decreased antigenicity of E_4 tumor cells by trypsin treatment in immune and normal BALB/c mice

Group	Treatment of challenge E_4 cells	No. of immunizations	Days after Challenge with Cells: 1 day, Immune	1 day, Normal	2 days, Immune	2 days, Normal
I	N[a]	2	2.04 ± 0.07[b,c]	1.41 ± 0.05	ND	
	T	2	1.58 ± 0.03[d]	1.34 ± 0.03	ND	
II	N	3	2.19 ± 0.09[e]	1.35 ± 0.07	2.03 ± 0.09[g]	1.44 ± 0.06
	T	3	1.72 ± 0.07[f]	1.47 ± 0.03	1.85 ± 0.07[h]	1.49 ± 0.02
III	N	4	2.55 ± 0.12[i]	1.45 ± 0.04	2.24 ± 0.08[k]	1.50 ± 0.04
	T	4	1.77 ± 0.09[j]	1.39 ± 0.03	1.70 ± 0.07[l]	1.42 ± 0.02

[a] N, nontrypsinized; T, trypsinized; ND, not done.
[b] Mean foot-count ratio ± S.E. (10 mice).
[c] c *versus* d, e *versus* f, i *versus* j, k *versus* l = $p < 0.001$; g *versus* h = $p < 0.05$.

Table 2

Regeneration in vitro of the E_4 cell surface antigens produced by X-irradiation after treatment with trypsin

Treatment of challenge E_4 cells[a]	Hr of Incubation in vitro after trypsinization: 1 hr	3 hr	6 hr	12 hr
N[b]	2.12 ± 0.06[c] (1.53 ± 0.04)[d]	2.23 ± 0.08 (1.51 ± 0.03)	2.35 ± 0.09 (1.52 ± 0.03)	2.18 ± 0.06 (1.48 ± 0.02)
T	1.70 ± 0.05[d,e] (1.55 ± 0.03)	1.69 ± 0.04[f] (1.48 ± 0.02)	2.07 ± 0.07[h] (1.52 ± 0.02)	2.28 ± 0.09 (1.55 ± 0.04)
T + 3000 R	2.27 ± 0.06[e] (1.54 ± 0.03)	2.19 ± 0.07[g] (1.50 ± 0.03)	2.54 ± 0.09[i] (1.45 ± 0.03)	2.38 ± 0.09 (1.54 ± 0.03)

[a] Trypsinized cells, whether subsequently X-irradiated or not, were inoculated within 1 hr after trypsin treatment.
[b] N, nontrypsinized; T, trypsinized.
[c] Mean foot-count ratio ± S.E. in immune mice.
[d] Numbers in parentheses, mean foot-count ratio ± S.E. in normal mice.
[e] e *versus* d, g *versus* f = $p < 0.001$; i *versus* h = $p < 0.02$.

pared with 1.65 ± 0.07 for the nonirradiated trypsinized cells. This result indicates that the regeneration of the cell surface antigens occurred during the period of incubation *in vitro* and not after subsequent inoculation of the cells into the footpad.

Dose Range of the Accelerating Effect Produced by X-rays. The effect of X-irradiation with doses ranging from 1000 to 8000 R on the antigenicity of trypsinized and nontrypsinized E_4 cells is given in Table 4. X-irradiation was performed immediately following harvesting of the E_4 cells from the cultured bottles by trypsin treatment or scraping; the cells were inoculated into the footpads of 4 times-immune mice within 60 to 90 min after the initial harvesting. Table 4 shows that the antigenicity of nontrypsinized E_4 cells was not increased by X-irradiation. In addition, the increased antigenicity at 60 to 90 min of the X-irradiated trypsinized cells usually equaled, but did not significantly exceed, the antigenicity of the nontrypsinized cells. In the other 2 separate sets of experiments in 2 or 3 times-immunized mice, X-irradiation with 3000 R consistently resulted in an increase in the antigenicity of trypsinized E_4 cells to normal levels, whereas X-irradiation of the trypsinized cells with 5000 and 8000 R had less or no effect.

Tumorigenicity of Nontrypsinized or Trypsinized E_4 Cells Exposed to Different Doses of X-irradiation. In order to study the relationship between effective irradiation dosages that increased the antigenicity of trypsinized E_4 cells and the dosages that inactivated the tumorigenicity of the tumor cells, 1 x 10^8 cells of nontrypsinized or trypsinized E_4 tumor after X-irradiation with 1000 to 8000 R were inoculated s.c. into syngeneic normal mice (Table 5). All mice given inoculations of nontrypsinized E_4 cells or nonirradiated trypsin-

Table 3

Inhibition of regeneration in vitro of the E_4 cell surface antigens by incubation with cycloheximide after treatment with trypsin and irradiation

Treatment of challenge E_4 cells[a]	Hr of incubation between treatment with trypsin plus irradiation and treatment with cycloheximide prior to footpad inoculation			
	0 hr	1 hr	3 hr	6 hr
N[b] + CY	2.34 ± 0.12[c] (1.30 ± 0.04)[d]	2.14 ± 0.12 (1.20 ± 0.03)	2.40 ± 0.13 (1.32 ± 0.04)	ND ND
T + CY	1.76 ± 0.04[e,e] (1.28 ± 0.02)	1.65 ± 0.07[g] (1.30 ± 0.03)	1.71 ± 0.08[i] (1.26 ± 0.05)	2.08 ± 0.06[k] (1.25 ± 0.04)
T + 3000 R + CY	1.88 ± 0.08[f] (1.41 ± 0.04)	2.05 ± 0.08[h] (1.27 ± 0.05)	2.30 ± 0.13[j] (1.23 ± 0.04)	2.42 ± 0.17[l] (1.27 ± 0.03)

[a] Trypsinized cells, whether subsequently X-irradiated or not, were inoculated within 1 hr after trypsin treatment.
[b] N, nontrypsinized; CY, cycloheximide; ND, not done; T, trypsinized.
[c] Mean foot-count ratio ± S.E. in immune mice.
[d] Numbers in parentheses, mean foot-count ratio ± S.E. in normal mice.
[e] i *versus* j = $p < 0.001$; g *versus* h = $p < 0.01$; e *versus* f, k *versus* l = no significant difference ($p < 0.5$).

Table 4

Increased antigenicity of trypsinized E_4 cells produced by X-irradiation in 4-times immunized mice

Treatment of challenge E_4 cells[a]	After 1 day		After 2 days	
	Immune	Normal	Immune	Normal
N[b] + None	2.40 ± 0.10[c]	1.36 ± 0.04	2.15 ± 0.09	1.38 ± 0.03
T + None	1.76 ± 0.08[d]	1.39 ± 0.03	1.70 ± 0.08	1.36 ± 0.02
N + 1000 R	2.36 ± 0.08[e]	1.47 ± 0.06	ND	
T + 1000 R	3.02 ± 0.24[f]	1.45 ± 0.05	2.10 ± 0.12[o]	1.30 ± 0.02
N + 3000 R	2.44 ± 0.09[g]	1.31 ± 0.05	ND	
T + 3000 R	3.18 ± 0.26[h]	1.43 ± 0.09	2.47 ± 0.11[p]	1.49 ± 0.02
N + 5000 R	2.59 ± 0.10[i]	1.42 ± 0.06	ND	
T + 5000 R	2.93 ± 0.29[j]	1.37 ± 0.05	2.06 ± 0.10[q]	1.50 ± 0.06
N + 8000 R	2.41 ± 0.11[k]	1.36 ± 0.06	ND	
T + 8000 R	2.11 ± 0.12[l]	1.48 ± 0.08	1.99 ± 0.09[r]	1.49 ± 0.04

[a] Trypsinized cells, whether subsequently X-irradiated or not, were inoculated within 1 hr trypsin treatment.
[b] N, nontrypsinized; T, trypsinized; ND, not done.
[c] Mean foot-count ratio ± S.E. (10 mice/point).
[d] f *versus* d, h *versus* d, j *versus* d, p *versus* n = $p < 0.001$; l *versus* d, f *versus* e, h *versus* g, o *versus* n, q *versus* n = $p < 0.02$.

Table 5

Tumorigenicity of nontrypsinized or trypsinized E_4 cells after different doses of X-irradiation in syngeneic BALB/c mice

Treatment of E_4 cells transplanted	No. of cells	No. of mice died/no. of mice used
N[a] + none	1×10^8	10/10
T + none	1×10^8	10/10
N + 1000 R	1×10^8	4/10 (10/10)[b]
T + 1000 R	1×10^8	2/10 (10/10)
N + 3000 R	1×10^8	0/10 (0/10)
T + 3000 R	1×10^8	0/10 (0/10)
N + 5000 R	1×10^8	0/10 (0/10)
T + 5000 R	1×10^8	0/10 (0/10)
N + 8000 R	1×10^8	0/10 (0/10)
T + 8000 R	1×10^8	0/10 (0/10)

[a] N, nontrypsinized; T, trypsinized.

[b] Numbers in parentheses, numbers of mice showing growth of tumors temporary/numbers of mice transplanted.

ized E_4 cells developed the tumors and died. Of 10 mice given inoculations of 1 x 10^8 nontrypsinized E_4 cells X-irradiated with 1000 R, all showed temporary growth of tumor, and 4 of the 10 mice died of lethal growth of the tumor. Similarly, in mice given inoculations of trypsinized E_4 cells, X-irradiation with 1000 R produced temporary tumor growth in 8 and lethal growth in 2. No mice given inoculations of either nontrypsinized E4 cells or trypsinized E_4 cells X-irradiated with 3000 to 8000 R showed any tumor growth.

The results show that X-irradiation dosages of 3000 R that were consistently more effective in increasing the antigenicity of the trypsinized E_4 cells appeared to be the minimum doses inactivating the tumor cells.

DISCUSSION

Effect of Trypsin on Cell Surface Antigens. Our results show that trypsin treatment of SV40-transformed 3T3 cells decreased their antigenicity as measured by the FPA. This is similar to the finding of Molinari and Platt (6), who demonstrated that polyoma virus-transformed cells treated with trypsin failed to induce a delayed hypersensitivity reaction against tumor-specific antigens as measured by footpad swelling. In addition, they reported that mice immunized with trypsin-treated normal 3T3 cells showed cell-mediated immune reactions against challenge with trypsin-treated normal 3T3 cells or nontrypsinized polyoma virus-transformed 3T3 cells but not with normal 3T3 cells. These results suggested that treatment with trypsin not only decreased but also modified their surface antigenicity. Ishimoto and Ito (5) also showed that trypsin removed the surface antigens of the papilloma cells induced by Shope papilloma virus. On the other hand, Brandchaft and Boone (1) have shown that trypsin treatment of cells from a Gross virus-induced rat lymphoma cell culture line resulted in a 3-fold increase, rather than a decrease, in the number of surface antigen sites. An increase in antigen sites of the cells was seen after treatment with as low as 0.025 mg trypsin per ml, and prolonged treatment with trypsin did not decrease the number of "exposed" surface antigens. Fakhri and Tan (2) reported that treatment of mouse plasmacytoma with 1% trypsin decreased the number of their surface antigenic determinants, whereas treatment with 0.1% trypsin did not, as measured by a rosette formation technique. It thus appears that the effect of trypsin on cell surface antigens differs with the cells used.

Accelerating Effect of X-irradiation on the Regeneration of Cell Surface Antigens after Trypsin Treatment. X-irradiation of trypsin-treated E_4 cells accelerated the regeneration of surface antigens to an extent that differed with the dose of X-ray used. The optimum accelerating dose was close to that which inactivated the tumor-producing potential of the E_4 cells. X-irradiation with 3000 R consistently induced greater regeneration of the cell surface antigens throughout all experiments.

At this time, we entertain 2 hypotheses regarding the effect of X-irradiation on the cell surface antigens resynthesis: (*a*) the X-irradiation accelerates the resynthesis of all cell surface membrane components more or less simultaneously, or (*b*) it specifically accelerates the resynthesis of the SV40 virus-induced cell surface antigen. Fogel and Sachs (3, 4) demonstrated that the frequency of virus-synthesizing cells in polyoma virus-transformed cells could be increased about 110-fold by X-irradiation. The highest rates of virus induction in the cells were observed by X-irradiation with doses of 2000 to 5000 R. Sabin and Koch (9) also found that X-irradiation enhanced the virus synthesis of the SV40 virus-transformed hamster fibrosarcoma cells. It was suggested that the cellular DNA is the target for induction of virus synthesis in these cells and that damage of the cellular DNA by physical and chemical agents results in activation of the virus genome. The important issue is whether the integrated viral genome of the cells exposed to X-irradiation is specifically induced to express the cell surface antigens.

The X-ray-induced enhancement of the regeneration of cell surface antigens in trypsinized cells was dose dependent with a threshold dose similar to the threshold dose for inhibition of cell division. Further experiments are needed to analyze the mechanism of these effects.

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