Killing of Cultured Rabbit Fibroblasts with Isoimmune Serum

Abstract. *A strain of cultured fibroblasts from a Flemish rabbit, when repeatedly injected into New Zealand rabbits, appears to induce the formation of antibodies. Incubation of the donor fibroblasts with these antiserums results in a significant reduction in the number of cells that survive and multiply.*

Antiserums that distinguish cultured cell strains obtained from different individuals of a single species would be useful as reagents in studying the somatic genetics of mammalian cells. Isoimmune serums (those obtained by injecting cells into animals of the same species) are preferable to heterologous antiserums as selective and discriminatory agents, since heterologous antiserums generally contain antibodies to antigens common to all individuals of a species. These antibodies could obscure differences in cell strains grown from different individuals and are selectively removed from antiserums only with difficulty or not at all. Cann and Herzenberg have recently shown that isoimmune serums could kill cells grown in culture (*1*). The cells killed were of an aneuploid line with round morphology derived from a mouse lymphoma. Antiserums to such cells were prepared by the injection of mice with cell suspensions obtained directly from mouse spleen and thymus, organs rich in lymphoid tissue.

It was of interest to us to see whether isoimmune serums could be obtained by injecting cells grown in tissue culture. In addition, we felt it important to determine whether killing with isoimmune serums could be demonstrated with fibroblasts, since it has been noted by several investigators that diploidy in cultured cells is associated with the fibroblast morphology (*2*). Diploidy is desirable in deducing from genetic analysis of cultured-cell strains information relevant to the donor. The rabbit was chosen as the experimental animal because of the ease with which rabbit cell cultures and large amounts of rabbit antiserum could be obtained, although previously reported attempts to demonstrate killing of cultured rabbit cells with isoimmune serums were not successful (*3*).

Table 1. Immunization histories of recipient rabbits. A "Freund's adjuvant" series consisted of a single subcutaneous injection of 2×10^6 cells suspended in complete Freund's adjuvant followed in 3 weeks by an intravenous booster of 2×10^6 cells. An intravenous series consisted of seven injections, total 1.0 to 1.5×10^7 cells, over a 10-day period. Each group of rabbits received three series.

Rabbit No.	Immunization series			Total dose, each rabbit ($\times 10^7$ cells)
	1	2	3	
	Group A			
173, 184 188, 196 198, 199	Freund's adjuvant	i.v.	i.v.	3.2
	Group B			
235, 236 239, 240 288, 289	i.v.	i.v.	i.v.	4.2

Twelve New Zealand rabbits were injected with a strain of cells freshly cultured from the skin of a Flemish rabbit (*4*). The cells were grown in medium 199 (*5*) supplemented with 15-percent pooled, heated, normal rabbit serum (New Zealand). The morphology of cells in the initial culture was variable; after the first subculture all cells were fibroblastic. Cells were harvested for injection and for killing experiments by extensive washing, suspending of the attached cells by incubation with trypsin-EDTA, washing, and resuspending in balanced saline. The immunization records of the 12 recipient rabbits are given in Table 1. After each series of injections, the serums obtained were analyzed for their capacity (i) to kill donor cells or (ii) to fix complement, or both; if the serums were negative by these criteria, the animals were given further immunization. All serums were handled so that the complement was retained (*6*).

To measure killing of cultured donor cells by antiserum, duplicate tubes containing 3200 cells in suspension were incubated for 30 minutes at 37°C with antiserum diluted 1 to 5. The reaction was stopped with 10 volumes of cold growth medium, and replicate plates were seeded and incubated. After 1, 3, and 6 days, respectively, samples of plates from each treatment were stained and the total number of cells attached to each plate was determined. This number was compared with the number obtained by treatment of donor cells with serum obtained from the same rabbit before immunization was begun (normal serum). The number of complement units in each serum used in the experiments was determined by hemolytic assay (*6*).

An intensive course of immunization with rabbit cells was required before killing antiserums could be demonstrated. In contrast, a modest course of immunization of rabbits with freshly cultured strains of human fibroblasts (*7*) or human cancer cells in long-term culture (*8*) produced killing antiserums of great potency. With the rabbit fibroblasts there was no evidence of significant killing in assays of serum after the first or second immunization series, irrespective of the type of immunization. Killing only became apparent after the third series. Six paired normal and immune serums (third series) were chosen at random for detailed analysis. In addition, pooled normal serum from the New Zealand rabbits was heated at 56°C for 30 minutes to eliminate complement activity. The heated serum was compared with the individual normal serums obtained prior to immunization to determine whether the normal serums had detectable natural antibody which, in the presence of complement, could kill the cultured fibroblasts from the Flemish rabbit.

Table 2. Effect on attachment of incubating trypsinized cells with immune and normal serums. Donor cells (3.2×10^3) in 0.2 ml saline G were incubated with 0.4 ml of a 1:5 dilution of the indicated serum in duplicate tubes for 30 min at 37°C. Then 6 ml of cold growth medium was added, and petri dishes were seeded with 1 ml portions of each incubation mixture. Replicate plates were incubated for 24 hours and stained, and the number of cells per plate was determined. Heated, pooled New Zealand rabbit serum, ΔRS. Complement units are expressed as $C'H_{50}$. The percentage killing is [1−(I/N)] 100. I, immune; N, normal.

Rabbit No.	Serum	$C'H_{50}$ per reaction tube	Average No. cells per plate*	Killing (%)
184	N	0.6	116 ± 18	
	I	0.7	19 ± 4	84
196	N	0.5	110 ± 7	
	I	0.7	51 ± 3	54
199	N	0	109 ± 7	
	I	0.9	66 ± 19	40
239	N	1.0	80 ± 7	
	I	1.3	8 ± 4	90
240	N	1.1	87 ± 26	
	I	1.0	80 ± 9	0
289	N	1.3	100 ± 17	
	I	0.8	107 ± 6	0
ΔRS		0	113 ± 11	

* From four plates.

The results of this experiment are given in Table 2. Rabbits 184, 196, 199, and 239 had produced killing antiserums (40 to 90 percent) relative to the corresponding normal serum; rabbits 240 and 289 had not. Little variation occurred in the attachment of cells resulting from incubation with the individual normal serums or the heated serum; this supports the interpretation that killing was induced by antiserums

to the donor fibroblasts. The data also show the reliability of using normal serum as a reference standard for killing in an isologous system, in contrast to the significant variation shown by normal serum when used as a standard in the analysis of heterologous cell lines (*8*). The data presented in Table 2 represent cells that survived treatment and were attached to plates 24 hours after seeding. To determine whether immune serum had any effect on the growth of surviving attached cells, sample plates from each reaction mixture were incubated for 3 and 6 days and stained, and the attached cells were then enumerated. The cells that survived killing by immune serums grew at the same rate as did those treated with normal and heated serums, and the percentage of cells killed, calculated from data at days 3 and 6, closely paralleled that at day 1. The variability evident in the immune serums and the fact that only four of six animals gave detectable response suggest that the antibody response was not the maximum obtainable. In addition, even with immune serums which could kill (third series serums), we could not detect antibody by complement fixation with donor-cell suspensions as antigen.

There are several possible interpretations for these observations. We may be dealing with genetic differences governing relatively few antigens, and these particular antigens may be only weakly antigenic. Another possibility is that cultured fibroblasts may be deficient in isoantigen molecules, or that their spatial arrangement prevents maximum exposure during immunization or killing experiments. We have not determined whether hyperimmunization will enhance the killing capacity of the antiserum, or the optimum concentration of complement necessary for maximum killing.

We conclude that the demonstration of killing of freshly cultured cells by isoimmune serums offers the possibility of utilizing antigenic markers in genetic analysis of these cell strains (*9*).

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References and Notes

1. H. A. Cann and L. A. Herzenberg, *J. Exptl. Med.* **117**, 267 (1963).
2. J. H. Tijio and T. T. Puck, *ibid.* **108**, 259 (195 → L. Hayflick and P. S. Moorhead, *Exptl. Cell Res.* **25**, 585 (1961).
3. P. B. Medawar, *Quart. J. Microscop. Sci.* **89**, 239 (1948); M. Allgower, T. G. Blocker, Jr., B. W. D. Engley, *Plastic and Reconstructive Surg.* **9**, 1 (1952).
4. D. A. Pious, R. N. Hamburger, S. E. Mills, *Exptl. Cell Res.*, in press.
5. J. F. Morgan, H. J. Morton, R. C. Parker, *Proc. Soc. Exptl. Biol. Med.* **73**, 1 (1950).
6. E. A. Kabat and M. M. Mayer, *Experimental Immunochemistry* (Thomas, Springfield, Ill., 1961), pp. 149–53.
7. D. A. Pious and S. E. Mills, in preparation.
8. R. N. Hamburger, D. A. Pious, S. E. Mills, *Immunology*, in press.
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X-Rays: Are There Cyclic Variations in Radiosensitivity?

Abstract. *When adult CF1 mice of either sex were x-irradiated at various times of day and under various conditions of light and dark, their responses showed no cyclic variations. Radiodiagnosis and radiotherapy may be practiced with equal safety at all times.*

Pizzarello *et al.* (*1*) recently reported that anesthetized rats, when treated with x-irradiation (to 900 r) at night, all died within 13 days, while other rats treated similarly in the morning all survived for more than 130 days. Untreated controls, irradiated at the same two times of day, also responded quite differently. Thus it was suggested that the biological responses of animals at 9 A.M. and at 9 P.M. are different, and that exposures at night are the more deleterious. This contention is of such importance to radiology that we have investigated the matter on a large scale, with variations in time of day as well as in the light-dark cycle (*2*).

A total of 2347 mice of two strains (CF1 and ICR) and both sexes were given x-irradiation under carefully controlled conditions, with and without prior anesthetization, at levels of exposure near the LD/50/30 range, in the morning and in the evening, to determine whether there might be any variations in radiosensitivity, as reported previously (*1*). There was no statistical evidence that x-ray treatment in the evening is more deleterious than the same treatment in the morning.

Mice irradiated at 6 A.M. did not survive quite as well as those exposed at 12 noon. This is explained on the basis of the metabolic activity associated with the nocturnal habits of these animals, which eat and mate at night and sleep during the day. There was no difference in the survival between animals irradiated in the morning and animals irradiated in the evening, or in animals irradiated before noon and animals irradiated before midnight.

The anesthetic sodium pentobarbital (Nembutal) afforded no protection to either males or females irradiated in the morning or in the evening, when they were exposed to supralethal doses of x-rays. There was high and rapid mortality at all times.

Since the biological variables are usually greater and more numerous than the physical variables in most experiments with x-irradiation, it seems obvious that all intrinsic and extrinsic variables must be eliminated or controlled and that large groups of animals are required to achieve statistically significant results involving lethality. With all variables except time of day properly controlled, there seems to be no evidence that mice are more radiosensitive in the evening than they are in the morning. In fact, to the contrary, our studies suggest that there are no cyclic variations in radiosensitivity—at least for mice.

Sexually mature males and females of the CF1 and ICR strains of mice were used. With the exception of one group of 14-month-old ex-breeder females, all were 3 to 4 months old.

The mice were kept eight in a mouse box (series 4 had six per box) under standard laboratory conditions of temperature, humidity, and daily fluctuations of light and dark, except where otherwise noted. After x-irradiation, daily checks of deaths were made.

The physical factors of x-irradiation were as follows: Parallel x-ray tubes, arranged so that the mice were exposed to a cross-fire, were run at 184 kv(peak), 30 ma, with 0.28-mm Cu and 0.50-mm Al filters having an aggregate half-value layer of 0.6 mm of Cu. In the first series the tubes were set at a target distance of 30 cm, with an air dose rate of 300 r/min, and in the later series the target distance was 28.5 cm, so that the air dose was 325 r/min. Depending upon the dose to be delivered, the exposures ranged from 2 minutes to 2 minutes and 10 seconds for the unanesthetized mice, and up to 3 minutes (975 r) for the anesthetized mice. All exposures were therefore acute. During exposure the mice were confined, five at a time, in a plastic box 13 cm in diameter and 4 cm high (inside measurements). The cover and sides were porous. The calculated dose in air approximated very closely the delivered dose to each mouse because the absorption by the plastic container