Cytotoxic Effect of Lymphocyte-Antigen Interaction in Delayed Hypersensitivity

Abstract. *Lymph node cells from inbred rats having delayed sensitivity to soluble proteins inhibit growth of syngeneic or allogeneic fibroblasts in the presence of specific antigen. A relation is suggested between this in vitro phenomenon and other systems believed to be specific manifestations of delayed hypersensitivity.*

Antigen-mediated inhibition of the migration of macrophages and fibroblasts has been related unequivocally to the delayed type of hypersensitivity as distinguished from humoral antibody formation (*1*). This inhibition affects normal macrophages and is caused by a substance released from sensitized lymphocytes reacting with specific antigen (*2*). An apparently unrelated manifestation of delayed hypersensitivity is the cytotoxic effect of lymph node cells from animals in the process of homograft rejection on cultured cells of graft donor origin (*3, 4*). In our study we have sought a common mechanism in these different in vitro phenomena.

Rat embryo fibroblasts (2×10^5) of the inbred Lewis, Brown Norwegian (BN) (*5*), or DA (*6*) strains were planted in 4 ml of complete medium (*7*) in 30-ml plastic flasks (*8*) 2 days prior to addition of sensitized lymph node cells and antigen. At the time of our study, these fibroblasts had been established, respectively, 1, 8, and 3 months previously (*9*), passaged two to four times, frozen in 1-ml volumes in 10 percent glycerol at −90°C, and passaged again several times before use. Each cell line was established from a single embryo and may be presumed to represent a line of identical genetic and antigenic constitution.

Adult male rats of Lewis and BN strains were injected in one footpad with 300 μg of heat-killed tubercle bacilli in Bayol F (*10*) or with 100 μg of hen-egg albumin in complete Freund's adjuvant (*11*). After 9 days cells were teased from the draining inguinal lymph node, washed once, and resuspended in complete medium. Viability of these "sensitized" cells varied between 75 and 90 percent. Normal cells were prepared from unsensitized rats in the same manner. Four milliliters of fresh complete medium containing sensitized or normal syngeneic or allogeneic lymph node cells plus tuberculin purified protein derivative (PPD) (*12*) or egg albumin were added to the fibroblast cultures set up 2 days earlier. Final concentrations of antigen ranged from 1 to 250 μg/ml and final numbers of lymph node cells from 0.25 to 2×10^7 (in 4 ml). After incubation for 24, 48, or 72 hours, the cell sheet was rinsed twice with Hanks's solution, and the remaining fibroblasts were harvested (*13*) and counted with an electronic particle counter (*14*). The greater cell volume of the fibroblasts compared to that of lymph node cells permitted separate enumeration (*4*).

Data from five representative experiments are given in Table 1. In the presence of tuberculin-sensitized lymph node cells from Lewis rats and 25 μg of PPD per milliliter, there was a marked reduction in the number of allogeneic (BN) fibroblasts surviving at 72 hours (experiment 1). No comparable effect was produced with PPD in the absence of lymph node cells or with PPD and normal cells, nor was any effect seen with sensitized cells without antigen or with added histoplasmin. The difference in survival of fibroblasts between control and experimental cultures was apparent at 48 hours but was much more striking at 72 hours (Fig. 1). Studies in syngeneic systems demonstrated that the cytotoxic effect was independent of a genetic difference between sensitized lymph node cells and target fibroblasts (Table 1, experiments 2 and 3). A strict antigenic specificity of the effect was established; lymph node cells from tuberculin-sensitized rats and rats sensitized to egg albumin killed target cells only in the presence of homologous antigen (experiments 4 and 5).

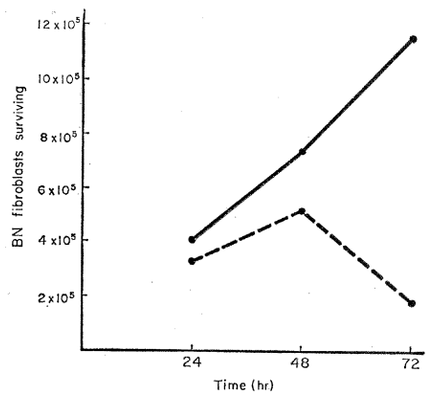

Fig. 1. Time course of the cytotoxic effect. Each point is the average of values from duplicate cultures. Antigen (12.5 μg of PPD per milliliter) and lymph node cells were added to all fibroblast cultures 2 days after they were set up. *Time* indicates number of hours after addition of lymph node cells and antigen; solid line, 2×10^7 normal Lewis lymph node cells; and dashed line, 2×10^7 Lewis lymph node cells sensitized to tubercle bacilli.

Each experiment in Table 1 has been repeated one to 12 times with variations in the ratio of lymphocytes to target cells and in concentration of antigen (Table 2). The cytotoxic effect has been completely reproducible. In both syngeneic and allogeneic systems and with both PPD and egg albumin, a significant effect was observed with final ratios of sensitized lymph node cells to target cells between 4 : 1 and 64 : 1, and a maximum effect at approximately 25 : 1. Both antigens were effective at concentrations of 1 μg/ml and gave maximum destruction of fibroblasts at 12.5 to 25 μg/ml. In some experiments as few as 10 to 20 percent of the fibroblasts survived. Sensitized lymph node cells from Lewis rats regularly produced a more intense cytotoxic effect on syngeneic and allogeneic fibroblasts than sensitized cells from BN rats did. A corresponding difference in delayed sensitization, as measured by skin test, was found between the strains in our experiments.

The data presented establish that the reaction of sensitized lymph node cells with antigen results in a cytotoxic effect on innocent bystander cells, in this case fibroblasts. The effect is specific, has a delayed time course, apparently does not require complement, and does not depend on a genetic difference between the two types of cells. The target cells need not be present during the primary reaction of sensitized lymphocytes with antigen; preliminary experiments show that this reaction, if carried out in the absence of fibroblasts for a period as brief as 1½ hours, can later lead to destruction of target cells.

The phenomenon described here resembles, in its quantitative aspects, the classical tissue culture experiment of Rich (*1*); its modern version, the capillary tube experiment (*2*); and the cytotoxic effect of lymphocytes on target cells in transplantation immunity (*3*).

Table 1. Survival of fibroblasts 72 hours after addition of soluble antigen (12.5 to 25 μg/ml) and lymph node cells (1.5 to 2 $\times$ 10^7). Each value is the average of three counts of duplicate cultures. Values obtained by adding nonspecific antigen (histoplasmin in experiment 1 and egg albumin in experiment 4) are given in parentheses. In experiments 1, 2, 3, and 5, percentage of survival was calculated as $\frac{1}{2}(f/c + f/e)$, and in experiment 4 as $f/(f)$. Lewis and BN are strains of rats; Tbc, tubercle bacilli.

Exp.	Origin of cells		Sensitizing antigen	Surviving fibroblasts ($\times$ 10^5)						Survival (%)
				No antigen added			Specific antigen added			
	Lymph node cells	Fibroblasts		*a* Fibroblasts only	*b* Normal lymph node cells added	*c* Sensitized lymph node cells added	*d* Fibroblasts only	*e* Normal lymph node cells added	*f* Sensitized lymph node cells added	
1	Lewis	BN	Tbc	8.9	9.2	9.5	8.3	9.1	3.2 (7.5)	34
2	BN	BN	Tbc	10.2	9.2	10.0	7.9	9.0	4.9	51
3	Lewis	Lewis	Tbc	7.6	9.6	8.3	7.7	10.3	2.6	28
4	Lewis	BN	Tbc	12.0	13.5		(11.2)	(16.0)	1.5 (14.2)	10
5	Lewis	BN	Egg albumin	12.0	13.5	14.0	11.2	16.0	6.8	45

We suggest that these in vitro phenomena all may depend on the same basic mechanism. In the Rich experiment, 1 μg of PPD per milliliter produced a slight cytotoxic effect, and 5 to 10 μg/ml, a maximum effect (*1*). Similar concentrations were effective in the capillary tube system (*2*) and in our experiments. In our system with soluble antigens, the effective ratios of lymph node cells to fibroblasts are the same as in experiments involving homograft cytotoxicity, in which ratios at the end of the culture period have varied between 1 : 1 and 100 : 1 (*3*, *4*).

The different experiments are also consistent in time course. Sensitized lymphocytes exposed to antigen produce a macrophage-inhibiting substance in less than 24 hours (*2*). In transplantation cytotoxicity, where the antigen is a fixed component of the target cells, initial specific "contactual agglutination" of sensitized lymphocytes and target occurs within the first few hours of culture (*3*). In our experiments, the initial reaction of sensitized lymphocytes with antigen takes place within a few hours. The time at which a secondary effect is observed varies with the nature of the system, that is, the species studied, identity of the target, and the physical relation of lymphocyte to target. Macrophage migration is inhibited within 24 hours, but cytotoxic changes are first seen at 24 to 36 hours and may occur later under altered culture conditions (*1*). Cloning efficiency of target cells may be affected in the homograft system within 24 hours, but grosser manifestations of cytotoxicity are not apparent until approximately 48 hours (*3*). In our experiments, a clear-cut cytotoxic effect was present by 48 hours.

These findings provide a possible explanation for cell damage that occurs, apparently nonspecifically, in the vicinity of reacting cells in the various types of delayed reactions (*15*). Elements as diverse as the activation and diapedesis of macrophages, the "invasive-destructive" lesion affecting the parenchyma in autoallergies and homograft rejection, and the vascular necrosis characteristic of very intense reactions may all be based on a common mechanism.

Table 2. Survival of fibroblasts 72 hours after addition of soluble antigen (12.5 to 25 μg/ml) and lymph node cells. Lymph node cells were obtained from animals of various strains (Lewis and BN) that had been sensitized to tubercle bacilli or to egg albumin. Ratio of lymph node cells to fibroblasts was calculated as number of lymphocytes added divided by number of cells at 72 hours in cultures that had fibroblasts only. Average percentage of survival obtained with nonspecific antigens (histoplasmin or egg albumin) given in parentheses.

Origin of cells		Ratio of lymph node cells to fibroblasts	No. of determinations	Survival (%)	
Lymph node cells	Fibroblasts			Range	Average
Lewis	Lewis	$<20:1$	5	26–78	46
		$\geq 20:1$	3	15–28	21
Lewis	BN	$<20:1$	12	10–90	56
		$\geq 20:1$	5	16–40	31 (95)
Lewis	DA	$<20:1$			
		$\geq 20:1$	2	54–67	60 (102)
BN	BN	$<20:1$	6	52–84	70
		$\geq 20:1$	5	36–69	58
BN	Lewis	$<20:1$	1	65	65
		$\geq 20:1$			

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References and Notes

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4. D. B. Wilson, *J. Exp. Med.* **122**, 143 (1965).
5. Microbiological Associates Inc., Bethesda, Maryland.
6. Courtesy of David Lubaroff from a gift of

Dr. Willys Silvers, University of Pennsylvania, Philadelphia.
7. Complete medium consisted of minimum essential medium (Eagle) with Earle's salts supplemented with 10 percent heat-inactivated fetal calf serum, 0.5 percent lactalbumin hydrolysate, 2 m*M* L-glutamine, and (per milliliter) 100 units of penicillin, 100 μg of streptomycin, and 0.25 μg of fungizone. All tissue culture reagents were obtained from Grand Island Biological Company, Grand Island, New York.
8. Falcon Plastics, division of B-D Laboratories, Inc., Los Angeles, California.
9. G. D. Hsiung, *Diagnostic Virology* (Yale Univ. Press, New Haven, Connecticut, 1964), p. 7.
10. *Mycobacterium tuberculosis*; Bayol F from Humble Refining Company, Bayonne, New Jersey.
11. Egg albumin (5× crystalline, Nutritional Biochemicals Corp., Cleveland, Ohio), 2 mg per milliliter of saline emulsified with equal amounts of complete Freund's adjuvant [15 percent Arlacel A (Atlas Powder Co., Wilmington, Delaware), 85 percent Bayol F, and 6 mg of tubercle bacilli per milliliter].
12. Parke-Davis, Detroit, Michigan.
13. Trypsin (0.25 percent) in a balanced salt solution containing 0.02 percent ethylenediaminetetraacetic acid.
14. Coulter counter, model A (Coulter Electronics Co., Hialeah, Florida).
15. B. H. Waksman, in *Mechanism of Cell and Tissue Damage Produced by Immune Reactions* (Second International Symposium on Immunopathology, 1961) (Grune & Stratton, New York, 1962), p. 146.
16. We thank Dr. Frank Ruddle for assistance in establishing the tissue culture lines. From a thesis submitted to the Graduate School of Yale University in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Presented in part at the meetings of the American Society of Microbiology, 1 May 1967, New York. Supported by NIH grants AI 06112 and AI 06455. N.H.R. was supported by PHS training grant 5 T1 AI 291.

29 May 1967

Inhibition of Lipolytic Action of Growth Hormone and Glucocorticoid by Ultraviolet and X-Radiation

Abstract. *Addition of catecholamines or growth hormone plus glucocorticoid activates lipolysis in isolated white-fat cells of the rat. If the fat cells were irradiated just before the addition of growth hormone and glucocorticoid, their lipolytic action was abolished. However, irradiation did not affect the activation of lipolysis by catecholamines.*

The addition of growth hormone (0.01 to 0.1 μg/ml) in the presence of glucocorticoid to free white-fat cells increases lipolysis after a lag period of 1 to 2 hours (*1*). The lipolytic action of growth hormone is abolished by inhibitors of protein synthesis, such as puromycin and cycloheximide, and by actinomycin which blocks DNA-dependent RNA biosynthesis (*1*). Catecholamines also accelerate fat-cell lipolysis, but their effect is rapid in onset and is not affected by any of these antibiotics (*1*). It is thought that catecholamines increase the concentration of cyclic 3′,5′-AMP (adenosine monophosphate) which activates the lipase involved in hydrolysis of triglycerides to glycerol and free fatty acids (*2*). Regulation of fat-cell lipolysis appears to be the major site for hormonal control of lipid mobilization and is thus of importance in the metabolic adjustments to caloric deprivation of animals.

Table 1. Effect of ultraviolet light and x-radiation on incorporation of leucine into protein and of uridine into RNA by fat cells, expressed as percent of inhibition. The values are shown as the mean ± S.E. of the percentage of inhibition due to ultraviolet light or x-radiation and represent seven paired replications. The basal value for incorporation of L-leucine-4,5-H^3 into protein was 1.26 percent of added label per gram of fat cells; for uridine-5-H^3, it was 1.39 percent. An average of 15 to 25 mg of cells were incubated for 4 hours in a volume of 2 ml.

Treatment	Inhibition (%)	
	Leucine into protein	Uridine into RNA
Ultraviolet (1 min)	50 ± 20	40 ± 14
Ultraviolet (2 min)	52 ± 20	70 ± 7
5000 rad	10 ± 16	80 ± 9
10,000 rad	27 ± 15	69 ± 10

We now report that ultraviolet light and x-radiation have the same effect as actinomycin and cycloheximide on the lipolytic action of hormones. The results support the hypothesis that RNA and protein synthesis are involved in the lipolytic action of growth hormone but not in that of catecholamines.

Free fat cells were isolated from the parametrial adipose tissue of starved female rats. The adipose tissue from three to six rats was incubated for about 45 minutes in Krebs-Ringer phosphate buffer containing 0.5 mg of crude bacterial collagenase per milliliter and 1 percent albumin. Samples of the dissociated adipose tissue were taken for exposure to ultraviolet light or x-radiation. Immediately after irradiation, the suspension of cells and tissue debris was centrifuged, and the medium with contaminating cells was removed by aspiration. The free fat cells were washed twice with albumin buffer and then placed in plastic flasks containing 3 percent bovine fraction-V albumin (Pentex No. 55) and 2.4 m*M* glucose in phosphate buffer; this cell suspension was shaken for 4 hours at 37°C in an incubator. The gas phase was air. Initial control values were obtained with fat cells incubated for 5 minutes, and all incubations were performed in duplicate.

Glycerol was analyzed by a fluorometric micromodification of the procedure of Vaughan (*3*) on samples of the medium and free fatty acids by a modification of the titrimetric procedure of Dole and Meinertz (*4*). The amount of fat cells present in each flask was based on total fatty acid content (*1*). Incorporation of L-leucine 4,5-H^3 (5 c/mmole) into protein and uridine-5-H^3 (5 c/mmole) into RNA was determined by a modification of the described procedures (*1*). However, RNA synthesis was based on uridine incorporation into material precipitable by trichloroacetic acid.

Fat cells were irradiated with a Picker x-ray generator operating at 280 kv and 20 ma. The beam was filtered with 0.4 mm of Sn, 0.25 mm of Cu, and 0.5 mm of Al. The dose rate was 1000 rad per minute, and the cells were placed in a plastic cup on a lead plate with air as the gas phase.

Ultraviolet irradiation was performed with a Hanovia No. 24,550 lamp which primarily emits light at 2537 Å. The cells were placed 33 cm below the light and exposed for 1 or 2 minutes. At this distance, 1-minute exposure inactivated 99.9 percent of a suspension of T3 bacteriophage, as based on plaque-forming ability.

Previously, a significant amount of basal lipolysis occurred, and the addition of bovine growth hormone (NIH-GH-B3 or B6) in the presence of 9α-fluoro-11β,17α,21-trihydroxy-16α-methyl-1,4-pregnadien-3,20-dione (dexamethasone, a potent synthetic glucocorticoid) accelerated lipolysis in fat cells from starved rats without added catecholamine (*1*). In these studies (Figs. 1 and 2), there was neither basal lipolysis by fat cells obtained from fasted rats nor lipolytic response to growth hormone and dexamethasone in the absence of added catecholamine. These differences are due to the finding that currently available commercial preparations of bovine fraction-V albumin contain an inhibitor of fat cell lipolysis (*5*). The effects of the inhibitor can be overcome by adding catecholamine; the increase in lipolysis due to growth hormone and dexamethasone was the same in the presence of either 0.5 or 0.05 μg of epinephrine per milliliter (*5*).

The results in Fig. 1 indicate that