CYTOTOXICITY MEDIATED BY SOLUBLE ANTIGEN AND LYMPHOCYTES IN DELAYED HYPERSENSITIVITY

I. Characterization of the Phenomenon*

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Delayed or cellular hypersensitivity underlies such different reactions as the delayed response to soluble protein antigens injected intradermally, the manifestations of certain autoallergic diseases, and the rejection of tissue transplants. The diversity of these in vivo manifestations is matched by the apparent diversity of in vitro phenomena shown in recent years to bear a relation to one or another form of delayed sensitization.

These include the original Rich and Lewis experiment showing inhibition by soluble antigen of macrophage and fibroblast migration (1–4); its modern counterpart, the capillary tube procedure of George and Vaughan (5); stimulation of macrophage maturation (6); release of endogenous pyrogen (7) or interferon (8) from macrophages after reaction with antigen; suppression of intracellular replication of bacteria or other microbial pathogens in "specifically sensitized" macrophages (9); uptake of antigen by sensitized lymphocytes (10); transformation of these lymphocytes into dividing blast-like cells (11, 12); the production of a specific cytotoxic effect by lymphocytes from animals with transplantation immunity on "target" cells of donor origin (13–17); and target cell damage produced by lymphocytes from animals with experimental autoallergies (18–20).

Until this time, no single mechanism has been recognized as responsible for these various phenomena. However, the recent studies of David and his collaborators (21–26), and Bloom and Bennett (27–30) suggest that a protein mediator, released from sensitized lymphocytes after interaction with soluble or cellular antigen, may produce secondary effects on other cells, inhibiting macrophage migration or altering their function in other ways (31–33). We have recently described a new system for the in vitro analysis of delayed hypersen-

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sitivity which involves the death of normal fibroblasts in the presence of sensitized lymphocytes reacting with specific antigen (34). In the present series of papers we present a complete description of this phenomenon and demonstrate some aspects of its mechanism. The data suggest a common pathway for the diverse in vitro manifestations of cellular hypersensitivity and for the tissue damage frequently associated with such reactions in vivo.

Materials and Methods

Rat Strains.—Rats were of three different highly inbred strains which differ at the Ag-B histocompatibility locus (35). Lewis and Brown Norwegian (BN) rats were obtained from Microbiological Associates Inc., Bethesda, Md. DA rats were derived from an original breeding pair provided by Dr. Willys K. Silvers, Department of Medical Genetics, University of Pennsylvania School of Medicine Philadelphia, Pa. Adult Lewis and BN rats were used for sensitization. Embryos of pregnant adult female Lewis, BN, and DA rats were used to establish fibroblast cell lines.

Sensitization.—Heat-killed, human type tubercle bacilli (Tbc) of the C, DT, and Pn strains (obtained through the kindness of Dr. I.S. Danielson, Lederle Laboratories, Pearl River, N.Y.) were ground to a fine powder and suspended in Bayol F (Humble Oil & Refining Co., Bayonne, N.J.); 300 μg in 0.1 ml was injected in a rear footpad. Delayed sensitization to egg albumin (EA) (5 × crystalline, Nutritional Biochemicals Corp., Cleveland, Ohio) and bovine gamma globulin (BGG) (Armour Pharmaceutical Co., Kankakee, Ill.) was induced by footpad injection of 0.1 ml of an emulsion containing 100 μg of the antigen in complete Freund's adjuvant (CFA) made up of equal parts of the antigen in saline and an oil phase containing 85% Bayol F, 15% Arlacel A (Atlas Chemical Co., Wilmington, Del.), and Tbc 6 mg/ml.

Culture Materials and Diluents.—All media and additives were obtained from Grand Island Biological Company, Grand Island, N.Y. The common diluent, unless otherwise specified was Hanks' balanced salt solution (HBSS). Complete medium consisted of Minimum Essential Medium (Eagle's) with Earle's base, containing 10% heat-inactivated fetal calf serum (56°C, 30 min), 2 mM L-glutamine, 0.5% lactalbumin hydrolysate, and antibiotic antimycotic solution (100 units penicillin, 100 μg streptomycin, and 0.25 μg Fungizone/ml). Madin-Darby solution (36) contained 8 g NaCl, 0.4 g KCl, 0.58 gm $NaHCO_3$, 1.0 g dextrose, 0.2 g versene, and antibiotic antimycotic solution (as above) in 1000 ml distilled water. The solution was sterilized by filtration and stored at −17°C. Viokase solution was prepared by adding 10 ml 2.5% Viokase solution to 90 ml Madin-Darby solution. Trypsin solution consisted of 10 ml 2.5% trypsin added to 90 ml Madin-Darby solution. Antigens were dissolved in complete medium before use. Purified protein derivative (PPD) was used either in tablet form (Parke, Davis & Co., Detroit, Mich.) or as a powder which was excipient and lactose-free (obtained through Dr. M. W. Fisher, Parke, Davis & Co.). Histoplasmin was obtained from Parke, Davis & Co.

Preparation of Lymph Node Cells.—Lymph nodes were removed, trimmed of fat, placed in warm (37°C) HBSS, and then transferred to a sterile glass Petri dish containing fresh HBSS at room temperature. The cells were teased out with needle rakes, centrifuged at 250 *g* for 10 min, washed once in fresh cold HBSS, and resuspended in complete medium at room temperature. Cell viability was determined with a 0.4% solution of trypan blue (Allied Chemical Corp., New York) in HBSS; cells unable to exclude the dye were counted as dead. The inguinal lymph node draining a footpad inoculation site 9 days after sensitization was employed as the "sensitized" node. "Normal nodes" were all lymph nodes from a nonsensitized animal.

Preparation and Maintenance of Fibroblasts.—Lewis, BN, and DA tissue culture lines were established according to the following method. Sterile technique was employed throughout.

Primary Explantation.—A single rat embryo, removed after approximately 15 days' gestation and freed of membranes, was placed in warm complete medium in a small glass Petri dish, washed thoroughly, and minced. The resulting brei was stirred magnetically at 37°C in 60 ml Viokase solution in a 125 ml Erlenmeyer flask. 20 ml of the supernatant was replaced after 10 min with fresh Viokase solution. At 20 min intervals, 40 ml of supernatant cell suspension was removed and replaced with 40 ml fresh Viokase solution, and the harvested cells were placed in centrifuge tubes containing 4 ml fetal calf serum at 4°C. This procedure was repeated three times. The cells were spun 10 min at 600 *g*. Three 250 ml flasks (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.) were seeded from each 20 min harvest in 20 ml complete medium (approximately 2–3 $\times$ 10^6 cells/bottle), and gassed with a mixture of 95% air, 5% CO_2. The medium was changed on alternate days by removing 10 ml old medium and adding 10 ml fresh complete medium.

Serial Transfer.—Stock cultures were transplanted once a week for use in assay or to maintain the stock. All medium (20 ml) was removed from each flask and 15 ml Viokase solution added. The flask was incubated at 37°C until the cells could be dislodged by gentle rocking (10–15 min). The suspension was centrifuged 10 min at 4°C and 600 *g*, and the cells resuspended 10 ml fresh medium and counted. 2 $\times$ 10^6 cells were seeded in 20 ml fresh medium in gassed 250 ml Falcon flasks.

Deep Freeze Storage.—After three to six transfers, a uniformly fibroblastic population predominated, as indicated by microscopic observation and Coulter counter mean cell volume determinations. Cells, harvested as described above, were prepared for freezing and storage by final resuspension in complete medium containing 10% glycerol. 5 $\times$ 10^6 cells in a volume of 1 ml were placed in a glass ampule (Kahlenberg-Globe Equipment Co., Sarasota, Fla.). The sealed ampule, after 30 min at 37°C, was brought slowly to −90°C in a Revco freezer (ultra-low temperature).

Recovery from the Frozen State.—To obtain a new supply of cells, an ampule was removed from the freezer and placed for a few min in a water bath at 37°C. The cells were then added to a 3 oz glass medicine bottle containing 10 ml complete medium and 5% CO_2 in 95% air at 37°C. After 1 wk, the cells could be transferred to 250 ml Falcon flasks and maintained as a stock or used in an assay.

Culture Protocols and Evaluation of Cytotoxicity.—

Plaquing technique: Falcon plastic tissue culture dishes, either 35 $\times$ 10 mm or 60 $\times$ 15 mm, were used. For histochemical observations 22 $\times$ 22 mm glass cover slips were placed in the dishes. 4–6 $\times$ 10^5 fibroblasts were added in a volume of 2 or 4 ml. Cultures were incubated in a moist atmosphere of 95% air, 5% CO_2. After 3 days the medium was changed and antigen solutions added. A drop of lymph node cell suspension (0.04 ml) was added to the fibroblasts in the incubator. When lymph node cells were added in this small volume and the cultures were not jarred, they settled in a small discretely circumscribed area. After 72 hr incubation, the medium was removed and the cell sheet rinsed twice with HBSS and fixed for 10 min in two changes of 100% ethyl alcohol. The cells were stained for 20 min in Giemsa (Harleco: Hartman-Leddon Co., Philadelphia, Pa.), rinsed in distilled water, air dried, and observed grossly and microscopically. Histochemical methods of observation are reported in a later paper of the present series.

Cell counts: 2 $\times$ 10^5 fibroblasts were planted in 4 ml complete medium in 30 ml Falcon flasks and gassed with 95% air, 5% CO_2. 2 days later the medium was replaced with 4 ml fresh medium, and lymph node cells and antigen were added in volumes of 0.5 ml and 0.1–0.2 ml of medium respectively. Further details are given in the tables describing individual experiments. The flasks were incubated at 37°C and harvested at various times, most frequently 72 hr. The medium, containing most of the lymph node cells and varying numbers of detached fibroblasts, was poured from each flask. The remaining cell sheet was rinsed twice with HBSS, incubated

TABLE I

Location and Viability of Fibroblasts and Added Lymph Node Cells at the Conclusion of Cytotoxicity Assays

Exp. No.	Lymph node cells	Total cells in medium and wash fluids	Viability of cells in medium and wash fluids	Fibroblasts in medium and wash fluids	Fibroblasts surviving in flask
	(150×10^5)	$\times 10^5$	%	$\times 10^5$	$\times 10^5$
1	Sensitized	—	—	0.89	10.2
	Normal	—	—	0.57	13.7
	0	—	—	0.12	14.9
2	Sensitized (L)	—	—	4.4	1.7
	Sensitized (BN)	—	—	1.54	4.96
	Normal (L)	—	—	0.8	5.2
	Normal (BN)	—	—	1.6	7.2
3	Sensitized	84	0	—	5.88
	Normal	44	0	—	11.94
4	Sensitized	115	7	2.29	2.37
	"	139	6	—	1.69
	"	128	9	—	2.94
	"	116	7	—	3.01
	"	106	3	—	4.94
	Normal	88	4	—	7.77
	"	98	7	—	7.04
	"	64	2	—	7.09
	"	83	9	—	7.42
	"	54	5	—	6.54
	0	1	0	—	5.36
5	Sensitized	45	0	2.2	5.0
	Normal	53	0	0.78	9.13
	"	27	0	0.73	9.37
6	Sensitized	39	—	3.49	3.53
	"	30	—	1.69	5.41
	Normal	30	—	0.85	9.47
	0	1	—	0.18	10.79

A variety of antigens and antigen concentrations were used in these experiments. Every culture received antigen. All cultures were harvested at 72 hr.

No cells remained attached to flask surface after trypsinization in any of these experiments.

for 15 min at 37°C in 2.5 ml trypsin solution, then pipetted vigorously to resuspend the cells uniformly. 1 ml of the final suspension was added to 19 ml counting fluid, and the cells were counted in a Coulter electronic particle counter (Model A, Coulter Electronics Co., Chicago, Ill.) at threshold 25 and aperture current setting 2. Preliminary studies with mixtures of lymph

node cells and fibroblasts showed that counts at this setting effectively excluded lymphocytes and debris and included fibroblasts. Three counts were made on each culture, and the values obtained for duplicate cultures averaged.

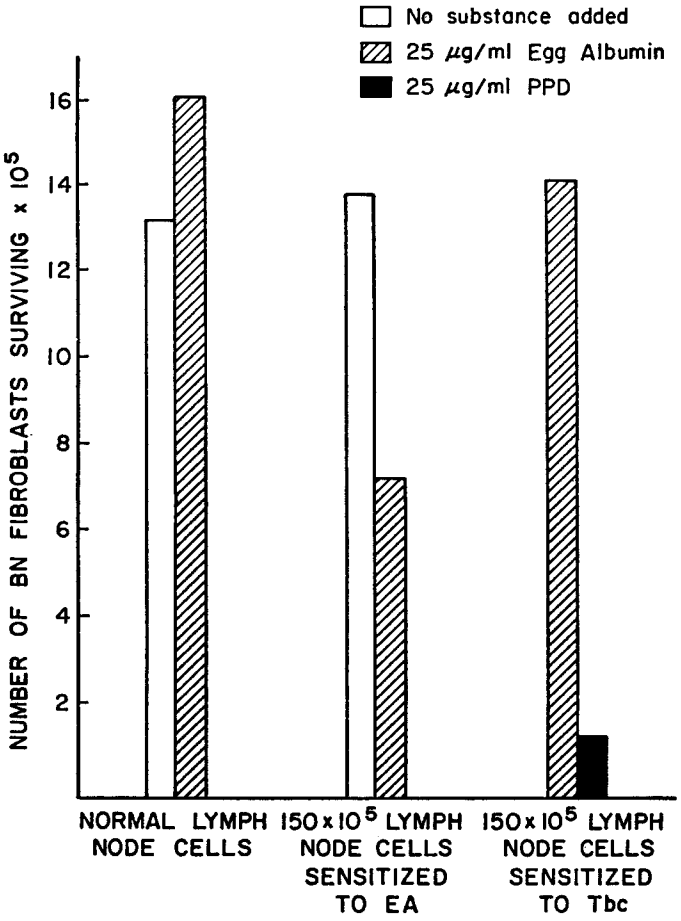

FIG. 1. Antigenic specificity of the cytotoxicity phenomenon. (Exp. 11, Table II). Survival of BN fibroblasts 72 hr after addition of Lewis lymph node cells and antigen.

RESULTS

Gross and Microscopic Evidence of Fibroblast Damage.—When lymph node cells from tuberculin-sensitized Lewis rats were added to fibroblast monolayers in the presence of PPD, an area of partial or complete clearing was seen (Fig. 3) which was independent of the genetic relationship between lymph node cells and fibroblasts. A comparable effect was produced neither by sensitized cells in the absence of antigen nor by normal cells in the presence or absence of anti-

gen. Microscopically, fibroblasts were seen to be vacuolated within 48 hr, and the lymphocytes adhered tightly to them. By 72 hr most lymphocytes were pycnotic, and damaged fibroblasts had become detached from the plastic (Fig. 4). Fibroblasts outside the cleared area appeared normal, as did those in cultures to which no PPD was added.

TABLE II

Antigenic Specificity of the Cytotoxicity Phenomenon

Exp.	Specific	Nonspecific	Surviving fibroblasts at 72 hr									Survival	
			No antigen added			Nonspecific antigen			Specific antigen				
			a	b	c	d	e	f	g	h	i		
			No lnc	Normal lnc	Sensitized lnc	No lnc	Normal lnc	Sensitized lnc	No lnc	Normal lnc	Sensitized lnc	i/h × 100	i/f × 100
			× 10^5	× 10^5	× 10^5	× 10^5	× 10^5	× 10^5	× 10^5	× 10^5	× 10^5	%	%
6	PPD	Histoplasmin	8.96	9.2	9.5	—	—	7.5	8.3	9.1	3.3	36	36
7	PPD	EA	6.7	9.1	10.2	6.7	8.2	10.4	6.7	8.4	5.0	60	48
8	BGG	EA	12.5	12.7	5.98*	11.9	11.7	7.1*	11.8	13.1	6.9	53	—
	EA	BGG	12.5	12.7	—	11.8	13.1	—	11.9	11.7	5.5	47	—
9‡	BGG	EA	8.4	11.98	10.98*	9.8	13.4	10.3*	10.0	14.2	9.6	68	—
	EA	BGG	8.4	11.98	—	10.0	14.2	—	9.8	13.4	4.8	36	—
10	EA	—	16.5	16.9	11.7	—	—	—	15.6	15.7	4.1	26	30§
		EA	16.5	16.9	16.3	15.6	15.7	16.1	13.9	16.7	6.7	40	40
11	EA	—	12.0	13.5	14.0	—	—	—	11.1	16.0	6.8	42	—
	PPD	EA	12.0	13.5	—	—	—	14.3	—	—	1.5	—	10
12	PPD	EA	4.7	6.5	—	4.6	6.8	4.3	5.1	5.8	1.3	22	30

150–200 × 10^5 sensitized lymph node cells (lnc) from Lewis rats immunized 9 days previously against the specific antigen. Source of fibroblasts varied.

* The "specific" antigen (BGG) is a component of the medium and is present in all cultures.

‡ BN lymph node cells.

§ Calculated as i/c.

The cytotoxic effect could be measured quantitatively by enumerating with a Coulter counter the living (trypsin-resistant) fibroblasts remaining attached to the flask at 72 hr (Fig. 1). In several similar experiments, the total of large cells in the supernatant medium and wash fluids and those surviving in the flask after trypsin-versene treatment was always lower in experimental than in

TABLE III

The Relationship of Antigen Concentration to Fibroblast Survival

Exp. No.	Target cells	Antigen added	Surviving fibroblasts in presence of			Survival c/b × 100
			a	b	c	
			No lnc	Normal lnc	Sensitized lnc	
		μg/ml	× 10^5	× 10^5	× 10^5	%
3	Lewis	0	—	9.8	10.0	102
		0.125	—	11.3	10.99	97
		1.25	—	10.3	8.06	78
		12.5	—	11.9	5.9	50
7	DA	0	6.7	9.1	10.2	112
		12.5	6.3	8.2	6.2	75
		25	5.8	8.4	5.0	60
1	BN	0	15.5	17.0	18.1	107
		1.25	15.4	19.7	12.8	67
		2.5	13.8	18.6	9.7	53
		12.5	14.3	17.2	6.3	36
4	BN	2.5	5.4	6.5	4.9	75
		12.5	5.6	7.3	3.6	41
		25	4.95	7.4	2.0	27
6	BN	0	8.96	9.2	9.5	103
		25	8.3	9.1	3.3	36
		50	5.3	7.96	3.3	41
11	BN	0	12.0	13.5	13.99	103
		3	12.8	14.8	8.1	54
		12.5	15.3	14.6	9.2	63
		25	11.2	16.0	6.8	42
10	BN	0	16.5	16.9	11.7	70
		250	15.5	15.6	4.1	26

Lymph node cells from Lewis rats injected with Tbc in oil or EA in CFA (Exp. 11 and 10). Cultures harvested 72 hr after addition of 150–200 × 10^5 lymph node cells and specific antigen (PPD or EA).

control flasks (Table I). The viability of cells not attached to the flask was very low and there was no preferential tendency for larger (i.e. fibroblasts) as opposed to smaller (i.e. lymphocytes) cells to be viable. No fibroblasts remained attached to the flask following trypsinization. Lymphocytes adherent to the fibroblasts were removed by trypsin-versene treatment and were easily distinguishable by the counter. When attached fibroblasts were counted at dif-

ferent times, a difference between experimental and control cultures was apparent by 48 hr and pronounced by 72 hr (34).

Antigenic Specificity of the Cytotoxicity Phenomenon.—A specific cytotoxic effect was also produced by lymph node cells from animals sensitized to antigens other than tuberculoprotein, when the sensitizing antigen was added to the culture (Fig. 1, Table II). Heterologous antigens produced no effect. Lymph node cells from rats sensitized to BGG killed fibroblasts in the *absence* of added antigen. However immunoelectrophoresis of fetal calf serum, a component of

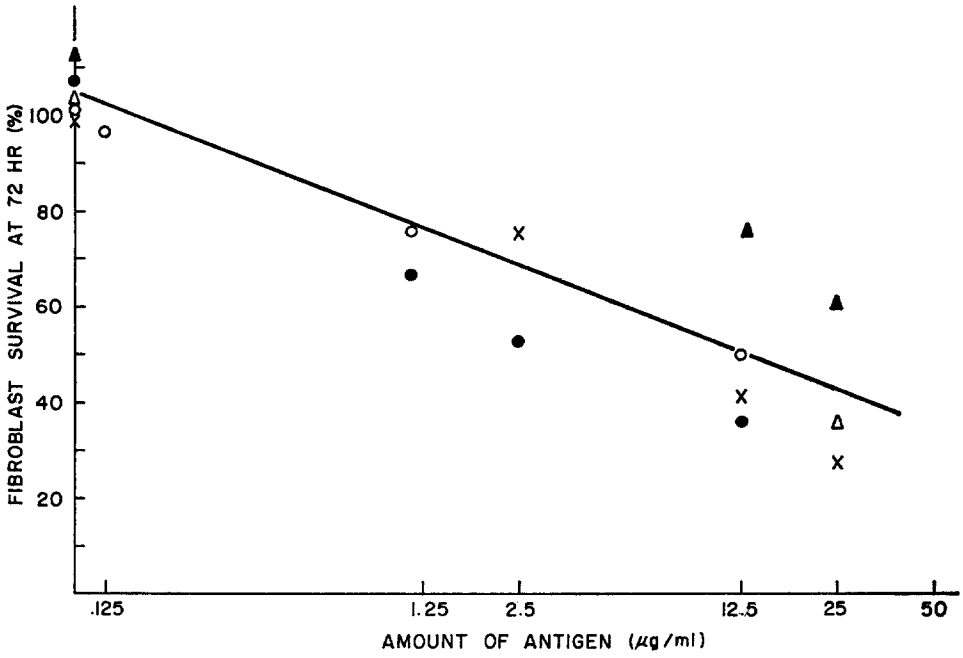

FIG. 2. Relationship of antigen, concentration, to fibroblast survival. Exp. 1, ○; 3, ●; 4, ×; 6, △; and 7, ▲; (See Table III). Line is best line through mean values.

the medium, showed the presence of a strong gamma component (see also Kniazeff et al., reference 37) and a component cross-reactive with BGG may have been present in the lactalbumin hydrolysate. Thus, cytotoxicity in this case probably also reflected a specific reaction.

Relationship between Antigen Concentration and Cytotoxic Effect.—A specific cytotoxic effect in the presence of sensitized lymph node cells was demonstrated with PPD and EA at concentrations of 1.25 and 3 μg/ml (Table III). PPD showed nonspecific toxicity at a concentration of 50 μg/ml (Exp. 6). However, excipient- and lactose-free PPD was effective in producing a specific cytotoxic effect (Exp. 4) and no nonspecific effect was produced by this antigen

TABLE IV

Relationship between Relative Number of Sensitized Lymph Node Cells and Cytotoxic Effect

Exp.	Source of cells		No. lnc	Surviving fibroblasts in presence of specific antigen		Survival b/a × 100
				a	b	
	lnc	fbl		Normal lnc	Sensitized lnc	
			× 10^5	× 10^5	× 10^5	%
13	Lewis	Lewis	25	5.8	4.6	78
			50	5.8	3.1	53
			100	6.9	2.3	33
			150	6.6	1.3	20
			200	7.2	1.1	15
14	Lewis	Lewis	50	6.0	5.0	84
			100	5.3	4.1	77
			200	6.6	2.6	40
15	Lewis	Lewis	45	9.4	6.8	72
			90	9.4	7.1	75
			180	9.4	5.8	62
16	Lewis	BN	50	4.6	3.0	65
			100	6.0	2.4	40
			150	7.4	1.9	26
			200	7.6	2.7	35
17	Lewis	BN	25	18.5	16.7	90
			75	20.2	14.7	73
			125	18.2	13.5	74
			175	17.6	12.0	68
18	BN	BN	25	12.2	10.2	84
			50	12.7	9.6	75
			100	13.0	8.8	68
			150	14.7	10.1	68
19	BN	BN	50	6.4	4.5	70
			100	6.8	4.3	63
			150	6.2	4.3	69
			200	7.4	4.9	66

Sensitized lymph node cells from rats 9 days after sensitization with Tbc (except Experiment 17, cells sensitized to EA).

Cultures harvested 72 hr after addition of lymph node cells and specific antigen.

alone. In general, the cytotoxic effect was proportional to the logarithm of the antigen dose in cultures where PPD was the specific antigen (Fig. 2). Though little work was done with antigen concentrations above 25 μg/ml, one experiment in which 250 μg EA/ml was employed resulted in greater fibroblast survival than would have been expected if the log-linear relationship had continued. This finding suggests that the system can become saturated with antigen.

The Relationship between Relative Number of Sensitized Cells and Cytotoxic Effect.—Addition of lymph node cells to fibroblast cultures in the absence of a specific immunologic reaction enhanced fibroblast growth (Table IV). The cytotoxic effect in the presence of specific antigen increased with increasing number of sensitized lymph node cells to a maximum, usually with 150–200 $\times$ 10^5 added cells. The magnitude of this effect was not influenced by the histocompatibility relationship between lymph node cells and target cells (syngeneic or allogeneic), though Lewis lymph node cells almost always produced a greater cytotoxic effect than BN lymph node cells (38). Cytotoxic effects were observed with lymphocyte to target cell ratios between 4:1 and 64:1, calculated as the number of sensitized cells added per number of fibroblasts surviving at the *end* of the experiment (in cultures with no added lymph node cells). A maximum effect was produced with ratios above 10 or 20:1. Since the fibroblasts doubled one to two times during the 72 hr culture period, actual ratios at the start of the experiment were approximately twice as high.

DISCUSSION

These experiments establish that sensitized lymph node cells from rats with delayed sensitivity to soluble protein antigens can interact with specific antigen and kill normal rat embryo fibroblasts within 48–72 hr. The effect is specific. It is produced with antigen concentrations as low as 1.25 μg/ml and with lymphocyte to fibroblast ratios between 4:1 and 64:1. In subsequent papers (38, 39), we will present data which show a close correlation of this in vitro effect with cutaneous delayed hypersensitivity and establish that its mechanism involves three steps: an initial rapid reaction of sensitized lymphocytes with specific antigen, release over several hours of a mediator, and death of fibroblasts caused by the mediator.

These findings provide a possible link between such seemingly disparate phenomena as inhibition of macrophage and fibroblast migration in the presence of soluble antigens (1–5, 21–33), specific cytotoxicity caused by lymphocytes from animals with transplantation immunity or one of the experimental autoallergies for target cells bearing the immunizing antigen (13–19), and blast transformation (11, 12). These are commonly considered manifestations of cellular or delayed type hypersensitivity. In each, one can identify a first immunologically specific step which involves antigen-lymphocyte interaction; a

second step which terminates in the release of a substance; and a third, immunologically nonspecific step which is highly variable depending on the nature of the target cell, the conditions of culture, and the parameter actually measured. The following quantitative comparison indicates that each of these has enough features in common with our experimental system to suggest a similarity of mechanism.

In our experiments a minimal cytotoxic effect was noted in the presence of 1.25 μg PPD/ml or 3 μg EA/ml and a maximal effect at concentrations between 12.5 and 25 μg/ml. The magnitude of the effect appeared to be linear with the logarithm of the antigen concentration over a certain range.

Sensitized lymphocytes are reported to undergo blast transformation after brief exposure to a PPD concentration of 1 μg/ml (40), and a unimodal dose-response curve is seen with this parameter of the reaction (41). Shea and Morgan (4) found that PPD exerted a cytotoxic and inhibitory effect on sensitized guinea pig splenic macrophages at a concentration of 1 μg/ml. No effect was detected in the presence of 0.1 μg/ml, and the effect was maximal at 5 and 50 μg/ml. Gangarosa et al. (43) found that concentrations of 5 μg, 50 μg, and 200 μg PPD/ml were equally effective in systems involving human cells. Similarly, Carpenter and Brandriss (42), using picrylated protein antigens, detected slight, but significant inhibition in the presence of 1 μg antigen/ml and maximal inhibition with 5 μg/ml. David et al. (21, 26) observed inhibition of macrophage migration in a capillary tube system with 0.75 μg PPD/ml, but routinely used 15 μg/ml.

Lymphocyte to target cell ratios found effective in our system were between 4:1 and 64:1 (see reference 34). An increasing cytotoxic effect was obtained by adding increasing numbers of sensitized lymphocytes. In the capillary tube system, a ratio of one lymphocyte to 100 target macrophages produces inhibition of macrophage migration in the presence of specific antigen (22). The greater effect of reacting lymphocytes in this case may depend on their close contact with the macrophages within the capillary tube; moreover, it is possible that the macrophage surface is highly sensitive to the inhibitory factor released by the lymphocytes. In homograft cytotoxicity experiments, the data of Wilson (15) and Brunner et al. (17) reveal effective ratios between 1:1 and 100:1. The log per cent survival of target cells was related linearly to the dose of sensitized lymph node cells, and Wilson interpreted this relationship as showing a 1:1 effect: one lymphocyte could kill one fibroblast (15). It is difficult to reconcile this conclusion with the suggestion that the cytotoxic effect may be mediated by a soluble factor (25, 27, 39). Perhaps the lymphocyte to target cell ratio expresses the amount of soluble factor required to produce a particular target cell effect.

Time course studies of the systems discussed here also suggest their similarity. In our system, the early specific step of antigen-lymphocyte interaction can

occur within ½ hour. A cytotoxic substance is released within 17 hr (39), and death of fibroblasts is clearly ascertained by 48 hr (34). Uptake of antigen by sensitized lymphocytes has been convincingly demonstrated by more direct techniques (10), and a 10 sec exposure of sensitized lymphocytes to PPD or tetanus-toxoid is reported to initiate blast transformation (41). In the system in which inhibition of macrophage migration occurs, the early antigen-lymphocyte interaction has not been analyzed separately. Release of the "migration inhibitory factor" occurs between 8 and 18 hr after addition of antigen (27), and inhibition of macrophage migration is seen at 24 hr. Cytotoxic changes in the macrophage, such as rounding up, detachment from the glass, and loss of phagocytic function, are observed within 24–36 hr (4, 45).

In homograft cytotoxicity, the early specific step is "contactual agglutination", a specific sticking of sensitized lymph node cells to target cells which contain the antigen, occurring within 1–3 hr (15, 20). If free lymphoid cells are removed, the attached lymphocytes cause as much destruction as the total population in undisturbed cultures (16). The period required for synthesis of a soluble factor has not been delimited. Sensitized lymphoid cells fail to destroy target cells placed on the other side of a Millipore filter perhaps because they are not in contact with antigen in the target cells (13, 14). A cytotoxic effect can be detected as early as 3 hr with such sensitive techniques as decrease in cloning efficiency (23). Morphologic changes are apparent by 18 hr (14); reduction in target cell number, however, is not significant until 24–48 hr after the addition of sensitized cells (15).

Allogeneic inhibition (47) had no apparent role in the experiments reported here; normal allogeneic lymph node cells caused fibroblast stimulation in all experiments. Moreover, sensitized cells in the presence of specific antigen did not cause a greater cytotoxic effect on allogeneic than on syngeneic targets. The possibility that a mutation in the Lewis fibroblasts led to formation of neoantigens, which would be recognized as foreign by sensitized Lewis lymph node cells and lead to some degree of allogeneic inhibition,was excluded by demonstration of the usual cytotoxic effect in the presence of tuberculin-sensitized Lewis lymph node cells and PPD with a new Lewis fibroblast line at the time of the first passage, before overgrowth by a mutated type could have occurred (unpublished data).

Holm and Perlmann's results with human lymphocytes (48) are related to the findings reported here. They report that lymphocytes stimulated by phytohemagglutinin and/or PPD kill various target cells and that this killing is related to the degree of blast transformation of the lymphocytes. They did not, however, investigate the specific phenomenon involving antigen in a syngeneic system. The recent discovery that culture supernatants from such stimulated systems cause blast transformation in normal lymphocytes (44, 49) implies that some of the blast cells observed in their study may have been *targets* of lymphocyte-antigen interaction rather than effectors.

SUMMARY

In the presence of specific antigen, lymph node cells from inbred rats with delayed hypersensitivity to tuberculoprotein, bovine gammaglobulin, and egg albumin produced progressive destruction of monolayers of rat embryo fibroblasts in tissue culture, first apparent at 48 hr and maximal at 72 hr. The effect was specific and did not depend on a genetic difference between the lymph node cells and target cells. It required antigen concentrations equal to or greater than 1.25 μg/ml and lymphocyte: target cell ratios of approximately 10 or 20:1. It could be evaluated both by a plaquing technique and by cell enumeration with an electronic particle counter.

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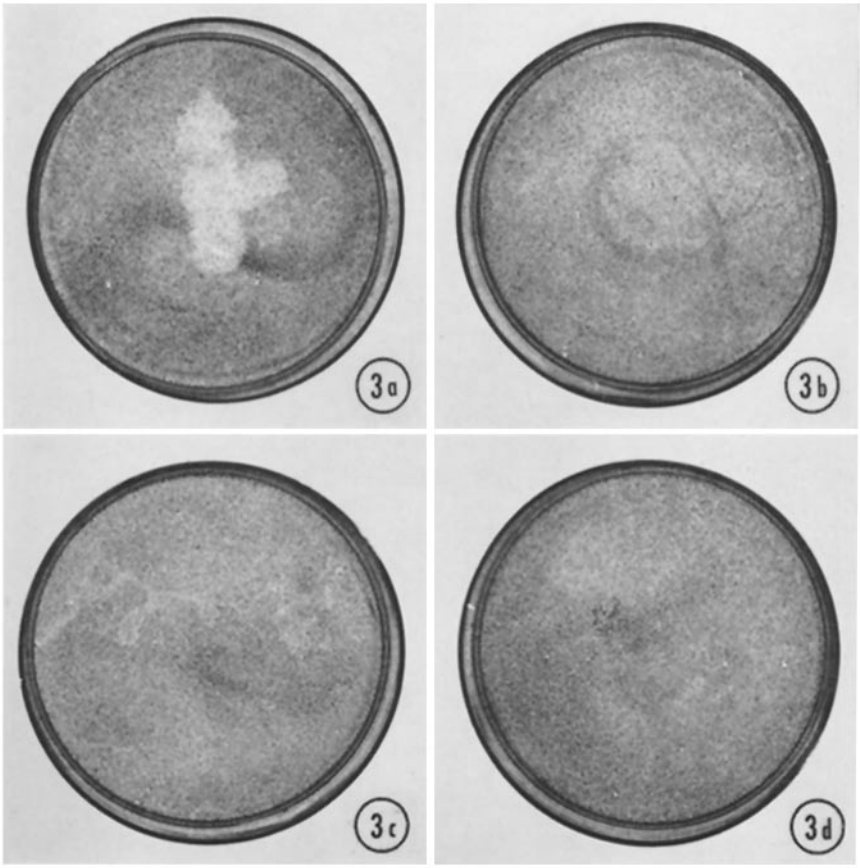

FIG. 3. Macroscopic appearance of Lewis fibroblast monolayers 72 hr after addition of lymph node cells and antigen. All plates were stained with Giemsa.

FIG. 3 *a*. 150 × 10^5 Lewis node cells sensitized to Tbc. 12.5 μg PPD/ml medium was present. × 1.

FIG. 3 *b*. 150 × 10^5 Lewis lymph node cells sensitized to Tbc. PPD was not present in medium. × 1.

FIG. 3 *c*. 150 × 10^5 normal Lewis lymph node cells. 12.5 μg PPD/ml medium was present. × 1.

FIG. 3 *d*. 150 × 10^5 normal Lewis lymph node cells. PPD was not present in medium. × 1.

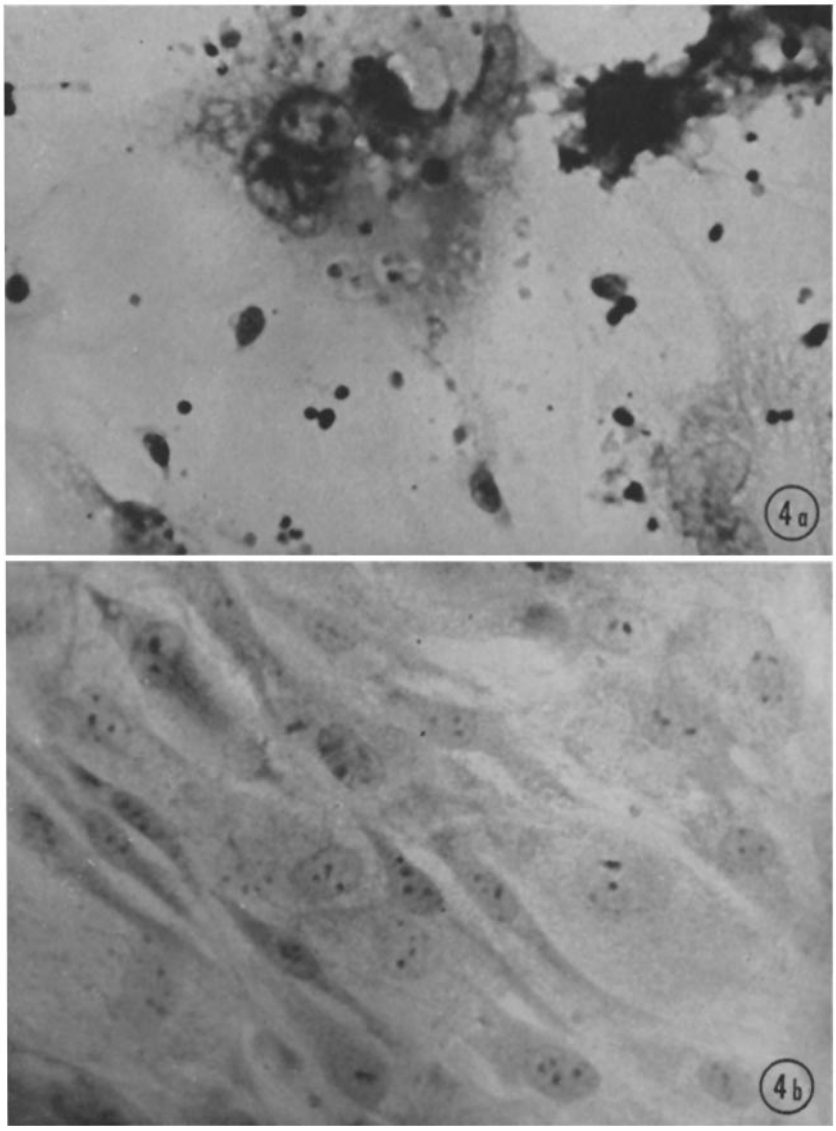

FIG. 4 Microscopic appearance of Lewis fibroblast monolayers 72 hr after addition of lymph node cells and antigen. Giemsa stain.

FIG. 4 *a*. Lewis lymph node cells sensitized to Tbc. 12.5 μg PPD/ml medium was present. ×625.

FIG. 4 *b*. No lymph node cells were added to these fibroblasts. 12.5 μg PPD/ml medium was present. ×625.