QUANTITATIVE STUDIES ON THE BEHAVIOR OF SENSITIZED LYMPHOCYTES IN VITRO

I. Relationship of the Degree of Destruction of Homologous Target Cells to the Number of Lymphocytes and to the Time of Contact in Culture and Consideration of the Effects of Isoimmune Serum*

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(Received for publication, March 19, 1965)

Numerous studies have implicated cells of the lymphoid series as agents responsible for the destruction of solid tissue homografts *in vivo* (1–5). This view has been substantiated by recent demonstrations that lymphoid cells (6–15) and macrophages (16, 17) procured from specifically immunized animals have a cytocidal effect on appropriate homologous "target" cells *in vitro*. It was previously reported that by the 6th to 7th day after grafting with homologous skin, lymphocytes from the regional lymph nodes of mice or rats had acquired the capacity to injure foreign target renal cells in culture, and that neither isoimmune serum nor complement was required for this destructive reactivity (15). The present communication is concerned with the application of a sensitive, reproducible *in vitro* assay system to elucidate further quantitative aspects of the destruction of homologous target cells by sensitized lymphocytic cells. Particular attention has been paid to the influence of (*a*) variation in the ratio of attacking lymphoid cells to target cells (*b*) the duration of contact with lymphoid cells and (*c*) the possible influence of the presence or absence of isoimmune serum on the cytocidal activities of sensitized lymphocytes.

Materials and Methods

Principle of the Test System.—Rats were immunized to the foreign transplantation antigens of a homologous strain. Cell suspensions consisting of known numbers of lymphocytes prepared from the lymph nodes of normal, *i.e.* non-immune, or sensitized animals were then added to previously "seeded" monolayer cultures of known numbers of target cells of donor strain origin. After a specified period of incubation, the number of target cells remaining in the

* This work was supported, in part, by United States Public Health Service Research Grant CA-05927 from the National Cancer Institute.

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culture was determined quantitatively. Adult rats, males and females 8 to 16 weeks of age, of the isogenic, albino Lewis strain and the agouti-colored DA strain[1] maintained at the Wistar Institute, were employed for this study.

Sensitization of the lymphoid cell donors was effected by means of bilateral orthotopic skin homografts on the lateral thoracic walls (18). For some experiments "hyperimmunized" DA rats were employed as lymphoid cell or serum donors; these animals were given subsequent bilateral subcutaneous inoculations, in the axillary regions, of approximately 100 million Lewis rat splenic cells 4 days after grafting with Lewis skin.

Serum was prepared according to standard techniques, using blood collected *via* cardiac puncture, from sensitized animals on the 8th day after immunization and, for control purposes, from normal donors. The serum was stored at −20°C. Before use, some samples were heated to 56°C for 30 minutes to inactivate complement; other experiments utilized fresh, unheated sera.

Homologous target cells were furnished by tumor cell lines developed from the two rat strains and arbitrarily designated as Le-1, Le-2, Le-3, and DA-1. Cells of the Le-3 line were derived from a spontaneously occurring Lewis mammary tumor;[2] Le-1 and Le-2 cells are two independently maintained sublines of a polyoma-induced kidney tumor originating from the same strain; DA-1 is also a polyoma-induced kidney tumor, derived from a DA rat. The virus-induced tumors were obtained from animals that had been inoculated subcutaneously at birth with 10^7 plaque forming units of polyoma virus.[3] Kidney sarcomas developed in inoculated animals within 3 weeks. These were removed aseptically and dissociated suspensions of cells were prepared with the aid of trypsin (19).[4] The cells were washed, suspended in medium,[5] and grown as monolayers in milk dilution bottles. The Le-3 cell line, prepared from the Lewis mammary tumor, was initiated in a similar manner. These lines of cells could be maintained in tissue culture by splitting 1:10 to 20 twice a week.

When employed as target cells, monolayers were dispersed into cell suspensions with a trypsin-versene mixture,[4] and then centrifuged at 150 *g* for 5 minutes. The pellet was then suspended in 5 ml of a growth medium, a sample taken for enumeration with a hemocytometer (0.2 ml cells mixed with an equal volume of 0.5 per cent trypan blue in phosphate-buffered saline and counted with a phase-contrast microscope) or a Coulter electronic particle counter (0.1 ml cells diluted to 10 ml with a counting fluid).[6] After appropriate dilution in medium,

[1] This line of agouti animals was obtained from Dr. T. T. Odell, Jr. at Oak Ridge National Laboratory, Oak Ridge, Tennessee, in its 11th generation of brother-sister matings. At F_{13} grafting tests showed them to be isohistogenic, *i.e.*, skin grafts exchanged randomly between members of the colony were permanently accepted. At present this DA strain is in its 19th generation of inbreeding.

[2] This spontaneous mammary carcinoma was found by Dr. W. L. Elkins; it was maintained in female Lewis rats by serial transplantation before it was adapted to tissue culture.

[3] The polyoma virus induced Lewis and DA tumors were supplied by Dr. V. Defendi.

[4] 0.25 per cent trypsin (Flow Laboratories, Rockville, Maryland) in Hanks' balanced salt solution containing 0.02 per cent disodium versene.

[5] The tissue culture medium consisted of 2 × Eagle's basal medium, 1 × Earle's balanced salt solution and 10 per cent calf serum (Baltimore Biological Laboratories, Baltimore). The antibiotics penicillin and streptomycin were added to a concentration of 100 μ/ml and 100 μg/ml, respectively. The serum was preheated to 56°C for 30 minutes before it was added to the media.

[6] The counting fluid was made up as 1 L of a 10 × stock solution containing NaCl 79.0 gm; KCl 3.7 gm; $Na_2EDTA \cdot 2H_2O$ 8.9 gm; Na_4EDTA 9.9 gm; 4 ml 0.5 per cent phenol red. One part of this stock solution was diluted with 9 parts distilled water and calf serum added to a final concentration of 0.1 per cent.

known numbers of the target cells (usually 5 to 10 × 10^4) were dispensed into Leighton tubes in a standard volume of 1 ml by means of a 2 ml Cornwall automatic syringe. These tubes were then incubated overnight at 37°C in a humid atmosphere of 95 per cent air to 5 per cent CO_2 during which time the cells firmly attached themselves to glass. For morphological studies, the Leighton tubes contained coverslips; otherwise, they were omitted. To inhibit cell division in some experiments, suspensions of target cells were X-irradiated with 2000 roentgens as before (15) and then seeded into Leighton tubes.

Preparation of Attacking Lymphoid Cells.—Lymphoid cell suspensions were prepared according to standard techniques (20) from the axillary and brachial lymph nodes of sensitized (8th postoperative day) and normal donors. These cells were washed and counted as described elsewhere (15), then suspended in medium at a known concentration. In some experiments, thoracic duct lymphocytes, obtained by a modification (21) of the technique of Bollman, Cain, and Grindlay (22) were employed as attacking cells.

Culture of Lymphoid Cells and Target Cells.—Following attachment of the target cells to the flat surfaces of the Leighton tubes, the media were discarded, and 1 ml of the lymphocyte suspensions added to each tube, after which the cultures were replaced in the incubator.

Enumeration of Target Cells.—The number of target cells surviving after a specified incubation period with normal or sensitized lymphoid cells was determined by one or, sometimes, two procedures: (*a*) by hemocytometer counts of stained nuclear isolates prepared with citric acid (23); or (*b*) with a model B Coulter particle counter[7] having an aperture of 100 μ.

RESULTS

Effect of Exposure of Homologous Target Cells to Various Numbers of Sensitized Lymphocytes; the Importance of Dosage.—To determine the extent of the destructive effect of sensitized lymphoid cells as a function of variation in number (dose) employed in culture, various dilutions of a suspension of normal or sensitized DA lymph node cells, in medium, were added to cultures of one of the three Lewis target cell lines. Leighton tubes containing target cells prepared the prevous day were arbitrarily assigned to several groups, each one having 3 tubes. The media were then discarded and 1 ml of a suspension of normal or sensitized lymph node cells (2.5 to 20 million) in medium was added to the first group; others received two-fold dilutions of the original lymphoid cell suspension, and the final group received medium alone.

For these tests, immune lymphoid cells were obtained from one of three sources: (*a*) the regional lymph nodes of animals sensitized by means of bilateral "target strain" skin homografts; (*b*) the thoracic duct lymph of similarly immunized donors; or (*c*) the lymph nodes of "hyperimmunized" animals. For some tests, non-dividing (irradiated) target cells were used. At the end of a 48 hour period of incubation with the lymphoid cells, the number of target cells remaining attached to the glass surface of the Leighton tubes was determined as described above.

The results of these various experiments, presented in Tables I and II, show that as the number of sensitized lymphoid cells in contact with homologous

[7] The author is indebted to Dr. Leonard Hayflick for the use of the Coulter Counter, obtained from Coulter Electronics, Hialeah, Florida.

TABLE I

Effect of Variation in Numbers of Sensitized and Normal Lymphoid Cells on the Survival of Homologous Target Cells in Vitro

System	Exp. No.	Mean No. ± SE × 10^3 and per cent target cells remaining after culture with various numbers of normal (N) and sensitized (S) lymph node cells																					
		Dose of lymph node cells (× 10^6)																					
		0			0.625			1.25			2.5			5.0			10.0			20.0		
		$\bar{x}$	$\pm SE$	%	$\bar{x}$	$\pm SE$	%	$\bar{x}$	$\pm SE$	%	$\bar{x}$	$\pm SE$	%	$\bar{x}$	$\pm SE$	%	$\bar{x}$	±	%	$\bar{x}$	$\pm SE$	%
Immune DA L.N. cells	1 N	467	31.3		536	21.5	93.4	544	15.1	77.8	520	15.1	60.6	537	14.4	39.5	551	34.6	17.6			
	S				501	13.1		423	11.1		315	9.3		212	14.1		97	15.4				
Le-1 target cells	2 N	387	15.1		479	34.0	98.3	602	16.5	87.7	515	40.1	58.1	499	36.2	41.5						
	S				471	23.4		528	29.9		299	24.2		207	11.8							
	3 N	324	7.0								390	20.3	43.1	385	28.1	25.2	401	1.3	8.2	209	10.2	0.6
	S										168	7.3		97	9.5		32	2.2		1	42.1	
Immune DA L.N. cells	4 *a* N	718	64.0		658	22.8	74.9	635	10.0	55.4	540	18.9	16.7	337	20.2	10.0						
	S				493	35.1		352	12.5		90	24.4		34	14.0							
	4 *b* N	750	21.0		726	12.0	92.9	755	45.0	52.3	612	33.6	22.2	352	27.2	7.4						
	S				675	37.2		395	32.8		136	6.5		26	5.6							
Le-2 target cells	5 *a* N	652	22.5		672	58.5	83.0	610	40.2	59.0	508	7.5	21.2	492	15.2	7.7						
	S				558	49.0		360	19.2		108	7.3		38	7.7							
	5 *b* N	655	29.6		652	19.3	95.5	705	94.2	55.0	532	57.8	30.1	450	16.4	7.1						
	S				623	45.9		388	21.0		160	2.3		32	8.0							
	6 *a* N	451	9.6		502	11.3	81.2	522	7.2	41.2	496	20.4	12.1	421	7.2	6.2						
	S				408	16.0		215	4.1		60	3.8		26	21.7							
	6 *b* N	436	17.3		554	58.8	61.4	477	49.6	44.2	556	65.2	8.3	409	28.4	1.3						
	S				340	15.6		211	43.2		46	4.0		5	1.4							
Immune DA L.N. cells	7 N	202	20.2		208	6.1	93.8	201	10.6	83.1	193	14.6	75.1	220	4.5	50.2	214	15.0	33.6			
	S				195	11.5		167	6.7		145	2.6		110	7.9		723	2.6				
Le-1 target cells (irrad.)	8 N	230	5.8		237	17.2	88.6	246	17.7	78.0	228	22.5	70.2	247	17.3	55.2	183	3.5	41.0			
	S				210	3.3		192	9.4		160	10.0		136	8.2		75	4.5				
	9 N	109	3.7								101	2.9	82.2	111	6.4	42.3	109	9.7	23.8	68	0.8	2.0
	S										83	4.4		47	5.3		26	5.5		1	0.0	
Immune DA L.N. cells	10 N	154	3.3								150	11.6	88.0	165	7.9	70.3	135	7.9	60.7	137	8.5	31.4
	S										132	3.8		116	5.9		82	6.6		43	6.0	
Le-3 (irrad.) target cells	11 N	165	3.3								147	3.8	86.4	146	4.0	81.5	149	4.2	65.4	117	4.0	28.8
	S										127	6.2		119	6.9		84	9.6		34	1.3	
Hyperimmune DA L.N. cells	12 N	183	2.8		275	31.0	86.7	216	4.0	81.5	277	10.6	51.4	307	11.4	43.0						
	S				195	4.0		176	8.0		145	5.6		132	4.0							
Le-1 target cells	13 N	632	16.0		712	35.5	99.4	664	25.3	95.8	704	16.0	86.9	900	18.3	49.3	912	29.0	22.4			
	S				708	9.8		636	20.8		612	13.4		444	31.5		204	19.6				
	14 N	592	61.2					796	50.9	75.0	806	66.0	63.4	1056	31.8	37.9	NT		18.5			
	S							596	41.6		540	24.0		400	16.9		172	16.9				
	15 N	369	16.9					501	42.4	98.0	527	44.0	90.9	655	58.8	67.3	652	34.2	15.0			
	S							491	30.2		479	33.7		441	52.8		982	24.0				
	16 N	349	17.0					407	21.5	98.3	493	9.0	82.6	623	26.5	58.6	632	16.5	37.3			
	S							400	24.0		407	3.0		365	42.4		236	8.0				

L.N., Lymph node.

TABLE II

Effect of Various Numbers of Normal and Immune Thoracic Duct Lymphocytes on Homologous Target Cells in Vitro

System	Exp. No.	Mean (± SE) No. target cells (10^3) remaining after culture with various numbers of normal (N) and sensitized (S) thoracic duct lymphocytes											
		Dose of lymphocytes ($\times 10^6$)											
		0	0.75	1.25	1.5	2.0	2.5	3.0	4.0	5.0	6.0	8.0	10.0	16.0
		$\bar{x} \pm SE$	$\bar{x} \pm SE$	$\bar{x} \pm SE$	$\bar{x} \pm SE$	$\bar{x} \pm SE$	$\bar{x} \pm SE$	$\bar{x} \pm SE$	$\bar{x} \pm SE$	$\bar{x} \pm SE$	$\bar{x} \pm SE$	$\bar{x} \pm SE$	$\bar{x} \pm SE$	$\bar{x} \pm SE$
Immune DA L.N.	17 N	608 ± 4.0		713 ± 13.3			760 ± 11.4			908 ± 30.7			956 ± 10.1	
	S			700 ± 14.4			724 ± 41.9			896 ± 28.3			960 ± 23.0	
Le-1 target cells	18 N	384 ± 33.5		412 ± 21.6			448 ± 5.6			516 ± 27.4				
	S			372 ± 17.0			508 ± 43.0			484 ± 23.0				
	19 N	348 ± 4.0	460 ± 20.0		496 ± 33.7			536 ± 17.2			532 ± 54.0			
	S		400 ± 37.5		416 ± 25.6			428 ± 17.4			372 ± 32.8			
	20 N	369 ± 4.0				603 ± 16.5			660 ± 28.3			747 ± 24.0		964 ± 21.9
	S					551 ± 40.8			473 ± 25.3			379 ± 17.9		291 ± 13.3

target cells is increased, fewer target cells survive. In general with unirradiated target cells *cytostatic* as well as *cytocidal* activities of the sensitized lymphoid cells were evident. Cells of the Le-2 line were more affected by immune lymph node cells than the other lines and there was more pronounced damage to

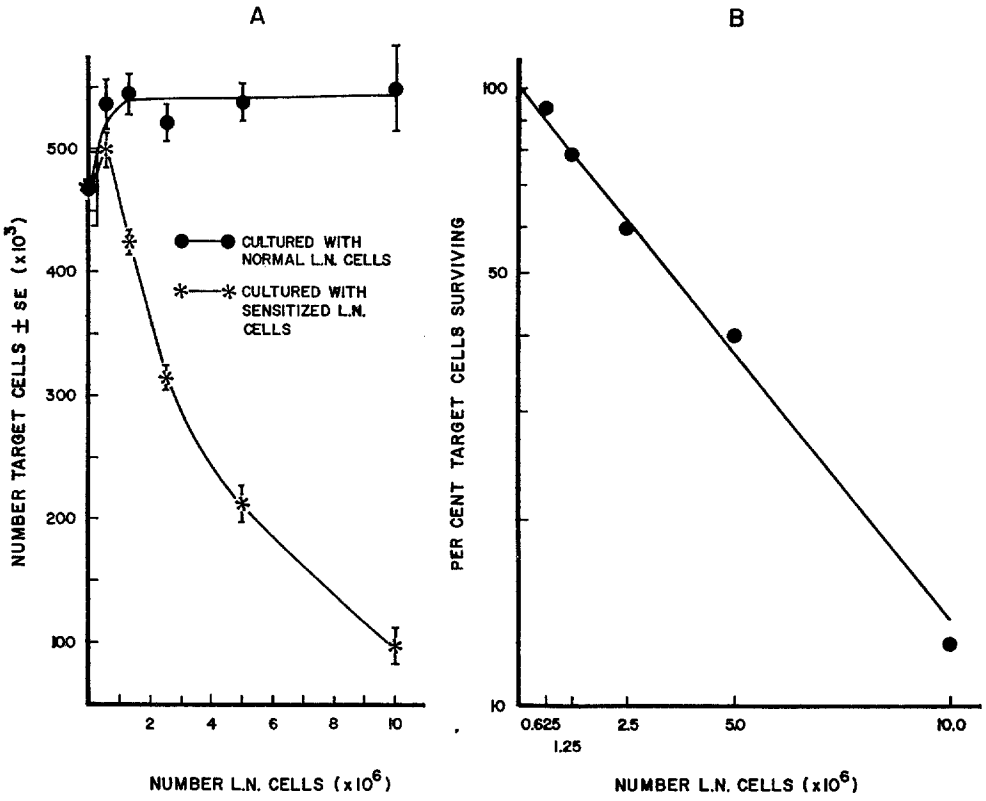

FIG. 1 *a*. Effect of various numbers of specifically sensitized or normal DA rat lymph node cells on Lewis target tumor (unirradiated) after 48 hours in culture.

FIG. 1 *b*. Semilogarithmic plot of the per cent target cells remaining after 48 hours culture (number surviving with sensitized lymph node cells × 100/number surviving with normal lymph node cells) *versus* the dose of lymphoid cells employed.

unirradiated Le-1 target cells than to irradiated Le-1 cells. Judged on the basis of the *per cent* of target cells remaining, "hyperimmune" lymph node cells were not noticeably more effective than node cells from "immune" donors. However, in experiment 14 a larger number of target cells was exposed to hyperimmune lymphoid cells, and on a cell for cell basis, the lymphocytes of this experiment must be considered to have been more active. Although thoracic duct lymphocytes were effective cytocidal agents, they were inferior to lymph node cells on

a numerical basis, and they did not become active until the 9th day after immunization. Further studies using lymphoid cell donors that have undergone different regimens of immunization might be fruitful.

For illustrative purposes, the results of two of these experiments (1 and 7) are presented graphically in Figs. 1 *a* and 2 *a*. These curves clearly show the

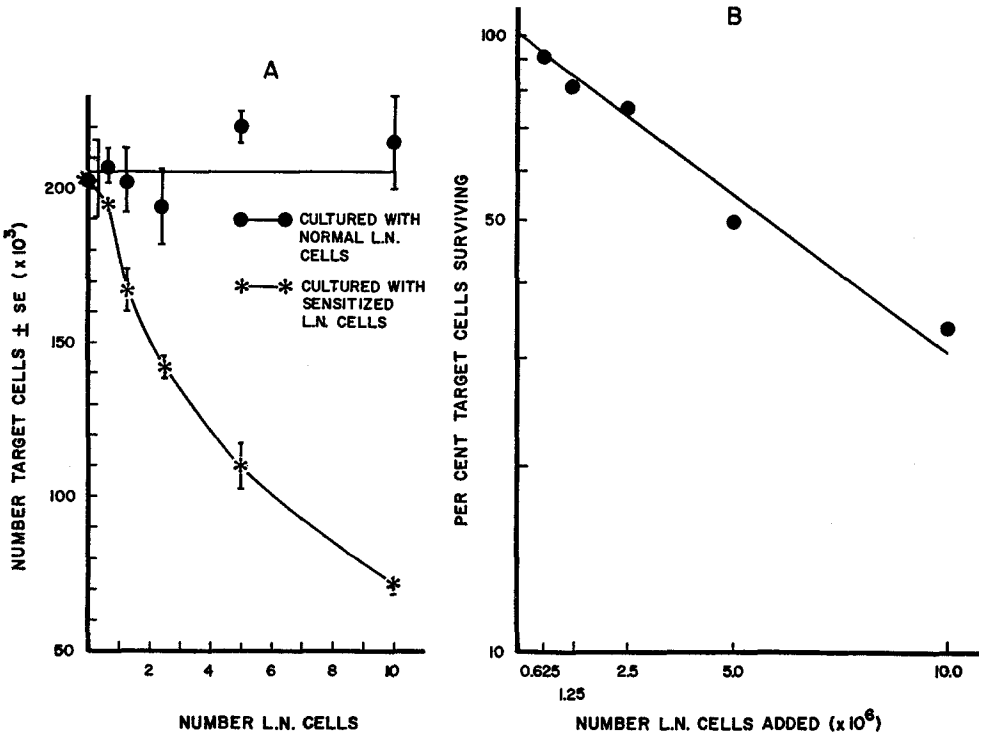

FIG. 2 *a*. Effect of various numbers of specifically sensitized or normal DA rat lymph node cells on Lewis target tumor cells (irradiated) after 48 hours in culture.

FIG. 2 *b*. Semilogarithmic plot of the per cent target cells remaining after 48 hours culture (number surviving with sensitized lymph node cells × 100/number surviving with normal lymph node cells) *versus* the dose of lymphoid cells employed.

inverse relationship which exists between the number of immunologically active lymphoid cells put into culture and the number of target cells which survive. Examination of the data on Table I reveals another response by the target cells to the presence of lymphocytes; normal lymphocytes or even relatively small numbers of sensitized lymphocytes cause an *increase* in the number of target cells as compared to the controls.

Quantitative Nature of the Destructive Influence of Sensitized Lymphoid Cells in vitro.—In attempting to define the response of target cells to varying doses

of lymphoid cells, it was noted that if the logarithm of the percentage of surviving target cells was plotted against the number of attacking lymphoid cells employed, the result approximated a straight line (see Figs. 1 *b* and 2 *b*). The application of appropriate statistical procedures for regression analysis and goodness of fit to these data confirmed that this relationship was linear.[8] The Y intercepts (A) and slopes (b) of these curves were determined from regression equations (Table III). A t test on the mean of the Y intercepts (104.19 per cent) indicated that it did not differ significantly from 100 per cent (*i.e.*, one). The results of these statistical procedures can be taken to indicate that the inverse relationship obtained between the dose of lymphocytes and the per cent of survival of target cells is an exponential one not unlike that found with "single-hit" inactivation phenomena (see reference 27).

Within any groups of similar experiments, the slopes (b) of the regression lines, related to the relative rate of decrease in the per cent of target cell survival (25), were generally consistent and afforded a comparative measure of the destructive capacity of various lymphoid cell suspensions.

The linear relationship between the log-per cent survival of the target cells *versus* the dose of lymphocytes, with the Y-intercept equal to 100 per cent indicates that, on the average, a single lymphocyte, if immunologically active, can destroy or at least have a detectably adverse effect on 1 target cell. Furthermore, provided that destructive contact between target cells and lymphocytes occurs on a random basis, these data are *inconsistent* with a premise that *two or more* activated lymphocytes are required to procure the destruction of a single target cell. If this is so, an estimate can be made of the proportion of lymphocytes which are immunologically "active"; *i.e.*, capable of destroying target cells under the conditions of the present test system. This estimate is made from the number of target cells present in the control cultures (N_{37}) divided by the dose of sensitized lymphocytes (X_{37}) which resulted in 37 per cent survival of target cells (See Table III). This latter figure is readily calculated from the regression equations. However, owing to the use of target cells capable of mitosis during the culture period with lymphocytes, it is difficult to obtain a precise figure for N_{37} to be used in calculating the per cent of "active" lymphocytes. The use of irradiated target cells obviates this difficulty. With such cells (experiments 7 to 9, 10, and 11) and the immunization procedure employed, *i.e.* grafting with homologous skin 8 days previously, it can be seen that approximately 1 to 2 per cent of the lymphocytes present in the regional lymph nodes draining the graft site display immunological activities *in vitro*.

[8] If the mean per cent survival of homologous target cells, Y, is related to the dose of attacking lymphoid cells, X, by the exponential expression $Y = Ae^{-cX}$ then the data of Table I should fit the straight line regression equation $\log_{10}Y = \log_{10}A + bX$, where A is the Y intercept and b the slope ($= -c \log_{10}e$). Analysis of variance as a test of linearity showed this to be the case. ($F^1_{18} = 196.113$, $P \ll 0.001$) (25, 26).

TABLE III

Summarized Data Compiled from Semilogarithmic Dose, Response Regression Curves Relating Per Cent Target Cell Survival to the Number of Lymph Node Cells Employed in Culture*

Experiment No.	System	Y intercept A	Slope $-b$ ($\times 10^{-6}$)	Dose of lymphoid cells resulting in 37 per cent survival of target cells X_{37} ($\times 10^6$)	No. target cells in control tubes N_{37} ($\times 10^3$)	Lymphoid cells "Active"
		per cent				*per cent*
1	DA L.N. cells	103.12	0.08991	5.10	540	10.6
2	Le-1 target	108.05	0.08716	5.37	490	9.1
3		87.25	0.10656	3.42	385	11.3
4 a	DA L.N. cells	86.57	0.20323	1.83	600	
4 *b*	Le-2 target	111.91	0.24440	1.98	675	
5 *a*		112.80	0.23860	2.03	575	
5 *b*		123.00	0.24851	2.12	580	
6 *a*		84.98	0.24713	1.44	515	
6 *b*		111.60	0.39412	1.21	500	
7	DA L.N. cells	92.71	0.04062	9.88	210	2.1
8	Le-1 target (x-irradiated)	87.21	0.03407	11.00	180	1.6
9		142.8	0.09069	6.49	110	1.7
10	DA L.N. cells	95.25	0.02339	17.66	135	0.8
11	Le-3 target (x-irradiated)	104.9	0.02807	16.46	120	0.7
12	DA L.N. cells ("hyperimmune")	92.96	0.07159	5.62	300	5.3
13	Le-1 target	118.61	0.07205	7.02	900	12.8
14		92.57	0.07248	5.53	1056	19.1
15		132.74	0.07063	7.89	650	8.2
16		108.90	0.04747	9.89	632	6.4
17 (7 da)	DA thoracic duct	—	—	—	—	—
18 (8 da)	Le-1 target	—	—	—	—	—
19 (9 da)		89.66	0.01782	21.71	500	2.3
20 (11 da)		100.33	0.03350	13.01	900	6.9

* Computed from Tables I and II.

Survival of Target Cells after Various Periods of Exposure to Sensitized Lymphoid Cells in Culture.—To provide information on the time needed for attacking lymphocytes to initiate and carry out their destructive activities against homologous target cells *in vitro*, a fixed number of normal or sensitized lymph node cells (5 million) was suspended in medium and added to several groups of Leighton tube cultures of target cells, each group containing 3 tubes; other

groups received medium alone. To facilitate morphological observations, some of the tubes contained coverslips. The target cells came from the Le-1, Le-2 or DA-1 lines; for some experiments Le-1 cells had been irradiated to inhibit cell division. At various times after addition of the lymph node cells, a group of tubes was withdrawn from culture and the mean number of target cells remaining was determined; parallel groups of tubes with coverslips were also withdrawn and the slips removed, dipped gently once in saline, fixed in methanol, and stained with May-Greenwald–Giemsa.

Morphological observations were confined to Le-1 cells, irradiated or non-irradiated, exposed to sensitized or normal DA lymph node cells. By the 7th hour of incubation, sensitized cells were seen to be aggregated around the target cells; but with little or no evidence of damage to the target cells. Only slight degrees of clustering of normal lymphocytes were seen at this time. Examination during the 20th to 30th hour showed that appreciable numbers of sensitized cells had adhered to the target cells. The normal lymphocytes could be easily detached from the target cells by gentle agitation, whereas the sensitized lymphocytes adhered more firmly and could not be removed. At this time, many of the target cells exposed to immune lymph node cells had begun to retract their cytoplasmic processes and assume a spindle or sometimes round form. Cytoplasmic areas with an eroded appearance, indicative of local destruction by lymphoid cells, were evident. Forty-eight hour cultures revealed essentially the same picture, except in more intense form. Fewer target cells were present in the cultures with sensitized cells and they were rounded in appearance and exhibited marked basophilia. Nuclear membranes were frequently fragmented and surrounded by "spilled-out", or lysed, nuclear contents. Few or no cells survived as long as 70 hours. Throughout this period normal lymphoid cells tended to aggregate and form clumps around target cells but with no concomitant destruction to the latter; the target cells in these tubes remained flat and healthy. Microscopic examination of irradiated target cells revealed one undesirable result of this treatment: a few of the cells began to develop fragmented or micronuclei. At no time, however, did more than a small percentage of the target cells have this appearance.

It was not possible to gain much information about the fate of the lymph node cells. Many were unavoidably lost in the saline wash prior to fixation and it was not possible to ascertain definitive nuclear detail in those that remained. Many of them appeared to have become pyknotic by 48 hours and sometimes only their fragments remained attached to the target cells by the 70th hour. Most frequently the target cells which showed the greatest damage were those that were heavily surrounded by the sensitized lymphocytes.

The results of the quantitative studies (presented graphically in Figs. 3 and 4 and summarized in Table IV) support the qualitative observations above. The data with non-irradiated cells are plotted as semilogarithmic growth

curves. The per cent of survival at a particular time (the number of target cells remaining after culture with sensitized lymphoid cells/the number remaining after culture with normal cells) in each experiment is plotted beneath the growth

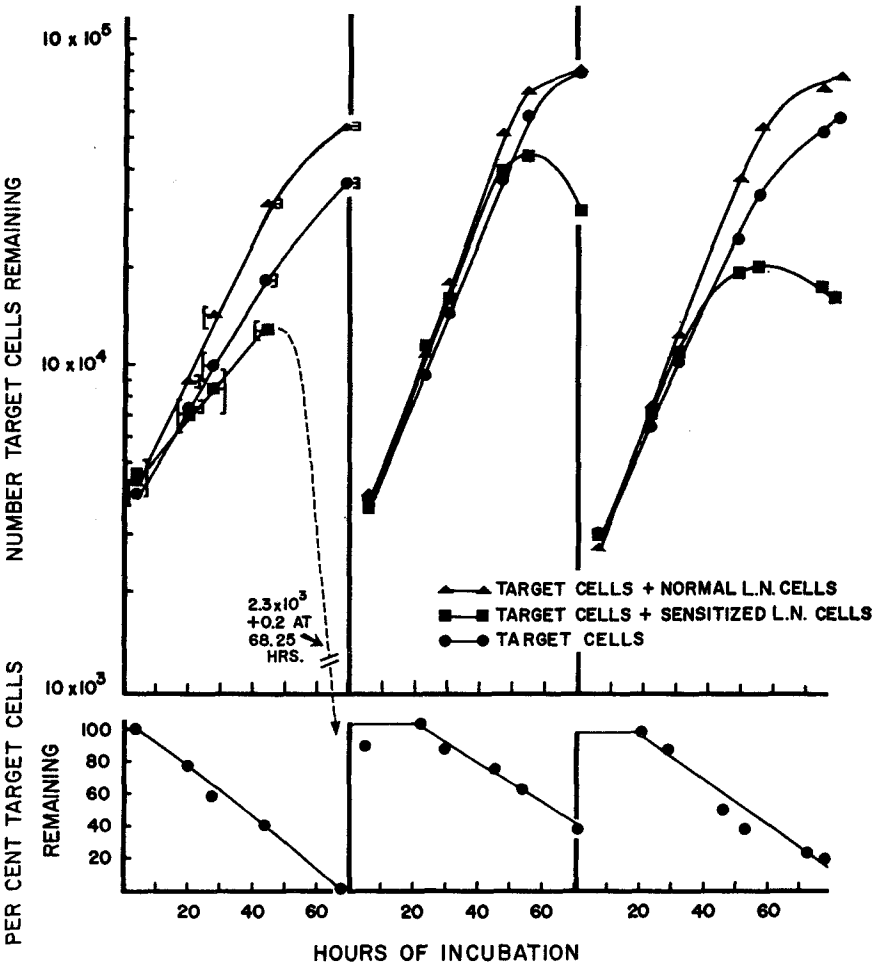

FIG. 3. Response of unirradiated target cells to normal or sensitized lymph node cells after various times in culture. Left and center: Lewis target cells plus DA lymph node cells. Right: DA target cells plus Lewis lymph node cells. The lower curves represent the per cent target cells remaining after various times.

curves. It can be seen that the number of target cells present at various times in cultures without lymphocytes follows a typical logarithmic growth curve with a doubling time (determined graphically) which is apparently characteristic of the cell line employed. When cultured in the presence of *normal* homolo-

gous lymphoid cells, the larger numbers of target cells present at any time reflect a faster logarithmic growth rate; the doubling time for these cells was consistently shorter than when grown in the *absence* of lymphocytes. Cultures exposed to sensitized lymphoid cells generally had a "latent period" of approximately 20 hours, *i.e.* it was not until this time that the immunological activities of the sensitized lymphocytes resulted in the presence of detectably fewer

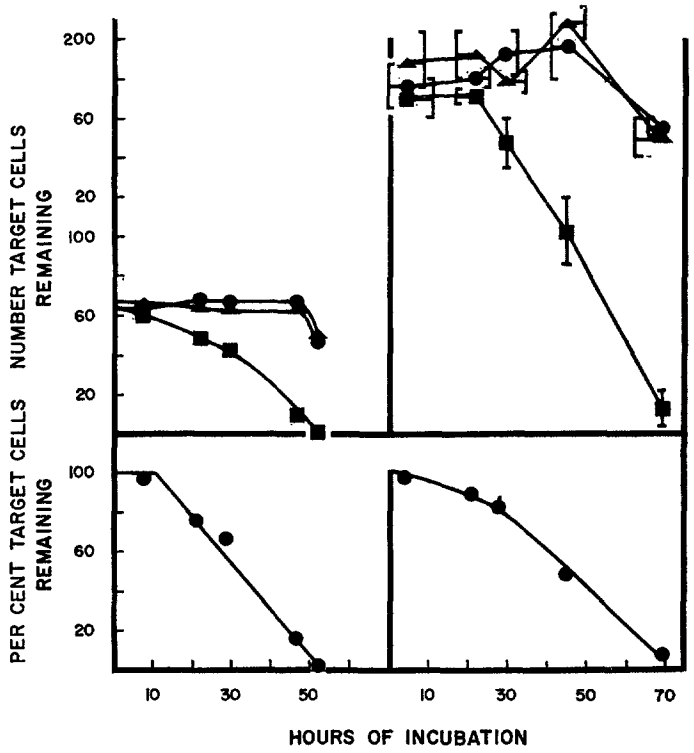

FIG. 4. Response of irradiated Lewis target cells to normal or sensitized DA lymph node cells after various times in culture. The lower curves represent the per cent target cells remaining after various times.

target cells, their number dropped steadily thereafter. When the *per cent* of target cell survival was plotted *versus* time, it was seen that the curves were nearly linear.

With irradiated target cells, cell division was greatly reduced and over a large portion of the culture period the number of target cells present in tubes without lymphoid cells or with normal lymphoid cells was more or less constant. Contact with sensitized lymphocytes for increasing periods of time resulted in a steady decrease in the number of surviving target cells.

Incubation of Lewis Target Cells with Preparations of Sonicated Normal or Sensitized Lymph Node Cells.—If, during the process of sensitization, lymphocytes acquire or produce a substance having an immunologic specificity which is responsible for the destruction of homologous target cells and, if this substance is contained within or bound to the lymphocyte, then sonic disruption may result in its release. Provided that this hypothetical substance can itself inflict damage upon homologous target cells, the present *in vitro* assay system should serve to detect and characterize it. To this end, large numbers of normal or sensitized DA lymph node cells suspended in medium and contained in

TABLE IV

Effect of Sensitized Lymph Node Cells on Homologous Target Cells after Various Times in Culture

Experiment	System		Latent period*	Doubling time*	
	L.N. cells	Target cells		No L.N. cells	Normal L.N. cells
			hrs.	*hrs.*	*hrs.*
1‡	Lewis	DA-1	20	14	11
2			22	15	12
3‡	DA	Le-1	5	19	14
4			19	19	15
5‡		Le-2	25	12	11
6			22	13	11
7‡		Le-1 (irrad.)	12	—	—
8‡			20	—	—

* "Latent Period" extrapolated from linear portion of per cent survival *versus* time curves; the doubling time determined graphically from log-survival *versus* time curves.

‡ Results presented graphically in Figs. 3 and 4.

plastic centrifuge tubes were sonicated in a raytheon sonic oscillator at maximum output (10 kc, 250 w) for variable periods of time. The resultant sonicates were then spun down at 250 to 1400 *g* in a refrigerated centrifuge (4°C), the supernates removed and diluted with an equal volume of media and added to cultures of Le-1 target cells. Microscopic examination of the preparations that had been sonicated for only 4 minutes showed that a few intact lymphocytes were still present, but none were found in preparations sonicated for longer periods. Although this procedure is crude, its success was anticipated in view of reports of the successful transfer of immunity *in vivo* by sonicated lymphoid tissues (28).

Microscopic examination of stained coverslip cultures of target cells exposed

to extracts of sonicated normal or sensitized lymph node cells revealed fewer target cells present than if cultured with medium alone, and these generally appeared to be in good condition. Some regions of the target cell monolayer were covered with a dark staining material which did not appear to have injured nearby target cells. No differences were discernible between cultures exposed to sonicates of normal or sensitized lymph node cells.

The quantitative results of this study (Table V) confirm the above observations and show, rather conclusively, that extracts of sensitized lymph node cells prepared by sonication are no more destructive to homologous target cells than similar preparations from normal lymphoid cells. Both are somewhat detrimental to target cell survival when compared to the effects of sonicated media.

TABLE V

Incubation of Lewis Target Cells with the Supernatant Fraction of Sonicated Lymph Node Cells Prepared from Normal or Immunized DA Rat Donors

No L.N. cells sonicated per tube of target cells ($\times 10^6$)	Duration of sonication	Centrifugation		Mean No. (±SE) target cells remaining after incubation with sonicated preparations		
		Speed	Time	Media alone ($\times 10^3$)	Normal L.N. cells ($\times 10^3$)	Sensitized L.N. cells ($\times 10^3$)
	mins	*g*	*mins*			
112	30	1400	5	372 ± 17.4	284 ± 10.1	300 ± 9.0
120	10	225	5	230 ± 5.8	123 ± 18.9	127 ± 16.0
105	10	925	5	184 ± 2.5	158 ± 6.2	145 ± 4.4
130	7	500	5	352 ± 24.0	396 ± 6.9	373 ± 24.0
65	4	225	5	709 ± 32.8	600 ± 11.3	612 ± 12.0

The Influence of Isoimmune Sera on the Cytocidal Reactivity of Sensitized Lymphocytes in Vitro.—The assay system described above was employed to determine the possible influence of immune sera on the *in vitro* reactivity of sensitized lymphoid cells. In particular, attention was focused upon the following questions: (*a*) does isoimmune serum *per se* have any cytocidal activity; (*b*) does the presence of specific isoimmune serum in the culture system confer any reactivity upon normal lymphoid cells; and (*c*) can isoimmune serum augment the destructive capacity of sensitized lymph node cells?

With these questions in mind, sera were collected on the 8th postoperative day from "hyperimmunized" DA rats and, for control purposes, from unimmunized, normal animals. Some of the sera used were fresh (unheated and unfrozen); others were frozen once or twice, and still others were heated to 56°C for 30 minutes to inactivate complement. These sera were used as a supplement to the replacement media added to Le-1 target cell cultures initiated the previous day in media containing heat-inactivated calf serum. Both normal and

sensitized lymph node cells were suspended in this medium at a concentration of 5 million per ml. Each experiment was repeated several times, each time with a different batch of immune or normal serum. In most cases, the serum of a particular batch was derived from a single donor.

Table VI, dealing with the influence of normal or isoimmune serum alone on homologous target cells, shows that heat-inactivated isoimmune serum is without any detectable detrimental effect and that fresh, unheated serum samples exhibit only a slight degree of cytotoxicity. In a single instance, however, there was a significant effect ($0.05 > P > 0.01$).

TABLE VI

Incubation of Lewis Target Cells with Serum from Normal or Immunized DA Rat Donors

Treatment of serum*	Concentration serum employed	Mean No. (±SE) target cells surviving after incubation		Difference‡
		Normal serum ($\times 10^3$)	Immune serum ($\times 10^3$)	
	per cent			*per cent*
△f1	40	217 ± 5.7	219 ± 17.2	
△	10	196 ± 6.1	207 ± 16.6	
△f1	10	260 ± 20.1	241 ± 11.0	
△f2	10	372 ± 17.4	364 ± 14.9	
△	10	455 ± 21.3	442 ± 33.2	
△	10	383 ± 22.5	393 ± 31.2	
	10	357 ± 18.8	321 ± 31.2	−10.9
	10	385 ± 26.8	367 ± 7.6	−4.7
	10	739 ± 44.8	624 ± 24.0	−15.6
	10	628 ± 17.2	616 ± 18.7	−1.9
f1	10	732 ± 15.2	632 ± 16.8	−13.7§

* △ Serum heat inactivated; f1, serum frozen once; f2, serum frozen twice.

‡ $100 - \frac{\text{Mean No. target cells surviving immune serum} \times 100}{\text{Mean No. target cells surviving normal serum}}$

§ Difference in means is significant ($0.05 > P > 0.01$).

To determine whether lymphoid cells could be made reactive, *i.e.* "quasi"-sensitized, by means of isoimmune sera, the Le-1 target cells were cultured with 5 million normal DA lymph node cells in the presence of isoimmune serum or, for the purpose of comparison, with serum from non-immune DA rats. After 48 hours exposure the number of remaining target cells was determined. The results, Table VII, show that neither fresh nor, with one exception, heated antisera from immune animals conferred any significant degree of destructive ability to "normal" lymphoid cells. The one unheated serum sample which did bring about a significant ($P < 0.01$) destruction of target cells came from the same batch which had some cytotoxic activity above. (See Table VI).

To determine the influence of the presence or absence of isoimmune serum on the cytocidal effectiveness of sensitized lymphoid cells, homologous Le-1 target cells were cultured with 5 million sensitized DA lymph node cells in immune or in normal serum supplemented (10 per cent) media. As before, the number of target cells surviving was determined at the end of a 48 hour incubation period. The results of these experiments (Table VIII) show that if heat–inactivated isoimmune serum had any influence at all on the destructive capacity of sensitized lymphoid cells, it was to slightly diminish that activity; at any rate, the presence of isoimmune serum did not augment the cytocidal capacity of sensitized lymph node cells.

The following experiment was performed to determine the effect of increasing

TABLE VII

Incubation of Lewis Target Cells with Normal Lymphoid Cells in the Presence of Normal or Immune Serum

Conditions of sera	Concentration of sera	Mean No. (±SE) target cells surviving after incubation with normal L.N. cells	
		Normal serum ($\times 10^3$)	Immune serum ($\times 10^3$)
	per cent		
Heated	10	271 ± 13.7	310 ± 13.7
		201 ± 8.9	207 ± 15.3
		254 ± 11.6	304 ± 18.8
		530 ± 29.3	466 ± 24.9
Not heated	10	692 ± 23.2	691 ± 17.6
		550 ± 40.0	449 ± 35.2
		924 ± 18.4	700 ± 28.0*
		872 ± 35.6	796 ± 24.4

* Difference in means is highly significant ($0.01 > P$).

the concentration of immune serum in the culture media on the destructive capabilities of sensitized lymph node cells. Normal or sensitized lymph node cells (2.5 million) were suspended in media containing 10, 20, or 40 per cent isoimmune serum and added to cultures of target cells. The number and per cent of target cells which survived a 48 hour culture period (Table IX) was found to be constant, regardless of the differing concentrations of immune serum; *i.e.*, increasing concentrations of isoimmune serum did not boost the cytocidal capacities of sensitized lymph node cells.

The Role of Complement in the in Vitro Reactivity of Sensitized Lymph Node Cells.—In spite of the use of heat-inactivated serum to supplement the culture media for the lymphocyte target cell mixtures, or as in some studies, the use of serum-free media, the possible participation of complement in the destruction

of homologous target cells *in vitro* by sensitized lymphoid cells cannot be denied entirely. It is conceivable that lymphoid cells contain or produce complement *in vitro* which might participate in their cytocidal activities.

To test this premise, assays for the presence of complement, using sheep

TABLE VIII

Incubation of Lewis Target Cells with Sensitized Lymphoid Cells in the Presence of Normal or Immune Serum

Conditions of sera	Concentration of sera	Mean No.(±SE) target cells surviving incubation with sensitized L.N. cells		Difference
		Normal serum (× 10^3)	Immune serum (× 10^3)	
	per cent			*per cent*
Heated	10	177 ± 3.2	216 ± 24.4	+22.0
		156 ± 2.2	186 ± 15.3	+19.2
		218 ± 8.7	240 ± 19.7	+10.1
		337 ± 21.3	409 ± 39.0	+21.3
Not heated	10	392 ± 24.4	381 ± 26.4	−2.8
		477 ± 25.2	495 ± 13.2	+3.8
		355 ± 5.6	443 ± 37.6	+24.7
		576 ± 18.4	720 ± 11.2	+25.0

TABLE IX

Incubation of Lewis Target Cells with Normal or Sensitized Lymph Node Cells in the Presence of Various Concentrations of Immune Serum

Concentration of immune serum	Mean No. (±SE) target cells surviving after incubation			Difference
	No L.N. cells (× 10^3)	Normal L.N. cells (× 10^3)	Sensitized L.N. cells (× 10^3)	
per cent				*per cent*
10	N.T.	310 ± 13.7	216 ± 24.4	69.7
20	N.T.	264 ± 16.2	196 ± 15.1	74.2
40	219 ± 17.2	273 ± 9.6	201 ± 9.7	73.6

erythrocytes as amboceptor in a microcomplement fixation test, were performed on the supernatant fractions of normal or sensitized DA lymph node cells that had been sonicated for 6 minutes as above and also on the media in which these cells were maintained for a period of 48 hours. Care was taken to omit serum from any of the preparative media. In no instance was lysis observed, a finding which indicates that at least one, if not all, of the complement components required for red cell lysis are not present in detectable quantities in lymph node cells. To ensure that sonication itself did not inactivate some component of

complement, guinea pig serum, diluted 1:10 with saline, was sonicated and used in the complement fixation test. Lysis was observed through a dilution of 1:80, showing that complement activity is not significantly diminished by sonication.

Effect of Separating Target Cells from Sensitized Lymphoid Cells by Means of a Millipore Membrane.—To determine whether or not mutual contact of sensitized lymphoid cells to homologous target cells was required for the cyto-destruction of the latter, double Rose chambers separated by an HA Millipore filter were constructed. Le-1 cells (50 to 100 thousand) were inoculated into 1 side of the chamber and medium on the other. The target cells were allowed to grow on the filter itself. Twenty-four hours later the media on both sides were replaced, on the target cell side with fresh media, and on the other side of the chamber with media in which normal or sensitized DA lymphoid cells were suspended (approximately 100 million). Chambers were taped to a vertical wheel rotating at 4 RPM and placed in a warm room at 37°C. An air bubble included on the lymphocyte side of the chamber facilitated agitation of the contents to promote diffusion of any substances that might be released by the lymphocytes across the membrane.

Forty-eight hours later, the chambers were disassembled, the lymphocyte side of the filter wiped clear on any adherent lymphoid cells, and the filter fixed in Tellyesniczky's fixative, stained with Mayer's hemalum and Papanicolaou cytoplasm stain (OG-6 and EA-50). After being cleared with xylol, the filters were mounted on glass slides with permount.

Examination of these stained preparations revealed a healthy population of target cells regardless of whether they had been exposed to diffusible products of normal or sensitized lymphoid cells. These results indicate that destruction of target cells by lymphocytes requires intimate contact between the two. However, the presence of an active diffusible material from sensitized lymphoid cells cannot be entirely ruled out since substances of high molecular weight may not have crossed the membrane in sufficient quantity to bring about any detectable destructive reaction.

DISCUSSION

The findings reported show that the *dosage* of lymphoid cells is one parameter of the degree of destruction inflicted upon homologous target cells cultured with specifically sensitized lymphoid cells. Survival of the target cells is inversely related to the number of immunologically activated cells present. Graphic plots of the percentage of target cells surviving, Y, *versus* the dose of sensitized lymphoid cells employed, X, show this inverse relationship is exponential and can be expressed in the general form (25):

$$Y = Ae^{-cX}$$

where A is a constant (the Y intercept) equal to 100 per cent and c is related to

the slope. Furthermore, the linearity of the semilogarithmic dose-response plots with a Y intercept equal to one (100 per cent) strongly suggests that a single lymphocyte, if immunologically active, is sufficient to destroy a single target cell.

With the present experimental design, the constant c ($= -b/\log_{10}e$) is dependent on several factors including the specific immunological activity of the lymphoid cell suspension, the vulnerability of the particular target cells selected, and the time of mutual contact between the two. When cells capable of rapid division were used as targets, the detrimental effect of lymphoid cells, reflected in the magnitude of the regression slope, was a composite of cytocidal and cytostatic activities on the part of the lymphocytes; *i.e.*, the outright killing of target cells as well as the inhibition of cell division. Only through the use of a non-dividing population of target cells was it possible to estimate the proportion of lymphocytes which could be considered "immunologically active" in terms of any lytic or cytocidal characteristics. It must be emphasized that the assay of immunologic activity, as used here, has meaning only in the context of the present experiments; *i.e.*, it is that quality of lymphocytes defined by their capacity to destroy or otherwise reduce the number of homologous target cells to which they are exposed over a specified period of incubation.

The finding that detectable reduction in the number of target cells cultured with sensitized lymph node cells occurs by the 20th hour of incubation is in line with the results of similar studies by others (9, 11), and with the observation that DNA synthesis is inhibited by the 10th hour (29). Increase in numbers of target cells exposed to normal lymphocytes, and even to sensitized lymphocytes for a short period of time, reflects a more rapid rate of cell division. This phenomenon has been described before with established lines of cells and is thought to be caused by the presence of "some growth promoting substance" (30) supplied by the lymphocytes. This higher rate of growth is confirmed by the finding that by the 5th hour of incubation with normal or sensitized lymphocytes, DNA synthesis is increased in target cells (29).

The aggregation of specifically sensitized lymphoid cells around target cells, which in the present studies occurred before the 7th hour of incubation, has also been observed before, and has been termed "contactual agglutination" (31). Although occasionally normal lymphocytes exhibit a similar clustering behavior, this must be considered as non-specific, since they can be dislodged by gentle agitation of the medium, whereas by the 5th hour of incubation sensitized lymphocytes cannot be removed by this procedure. Moreover, this contactual behavior of specifically sensitized lymphocytes is relatively temperature independent; it occurs at 37°C with subsequent target cell destruction, and also at 27°C, although at the latter temperature no destruction of target cells follows (32).

Attempts to implicate a serum component or a soluble factor in the destruction of target cells by lymphocytes were unsuccessful, but were nevertheless, informative.

(*a*) Separation of target cells from sensitized lymphoid cells by Millipore filter prevented any destruction of the target cells.

(*b*) Cytocidal activity could not be recovered from sonically disrupted sensitized lymph node cells.

(*c*) Isoimmune serum, in the absence of complement, was apparently not sufficient in itself to bring about the destruction of target cells. When used in "reconstruction experiments" similar to those of Rosenau (8) and Koprowski and Fernandes (31), preparations of immune serum failed to confer cytocidal reactivity upon otherwise *normal* lymphoid cells. In fact, even when used in conjunction with *sensitized* lymphoid cells, if isoimmune serum had any effect at all, it was to slightly inhibit this activity. Such a system might therefore serve as an experimental *in vitro* model for the study of enhancement.

(*d*) By design, the media can be disregarded as a source of complement activity. So too, can the lymphoid cells themselves, since detectable complement activity could not be recovered from sonically disrupted lymph node cells, nor from the media in which the lymphocytes were maintained for 2 days. The reason for considering whether complement is essential for the destructive activities of sensitized lymphocytes stems from the well recognized role of the various components of complement in the immunodestruction of cells or tissues. If complement is not required for the cytocidal activity of sensitized lymphoid cells it follows that lymphocytes from an immune animal can be considered as agents capable of killing target cells, probably by means not involving "conventional" methods of immunodestruction in which a major role is played by antibody and complement.

One hypothesis concerning the mechanism of the destructive interaction of sensitized lymph node cells and target cells that is consistent with the results of the present studies and those of others is as follows: when specifically sensitized lymphocytes are confronted with homologous target cells, destruction of the latter results *via* a two step process. First the attacking lymphocytes attach to the targets; the attachment being mediated by a non-toxic cell-bound factor having an immunological specificity which serves merely to ensure intimate contact between the lymphocytes and target cells. Then, the destruction of the target cells follows; this process being dependent on the metabolic activity of the lymphocytes that are now attached.

SUMMARY

When lymphoid cells, derived from rats immunized with respect to homologous skin, were cultured with target cells originally of donor origin, cytocidal and

cytostatic activities of the attacking lymphocytes became evident. By application of a sensitive and reproducible quantitative assay system, various aspects of the mechanism of this destructive interaction between target cells and lymphocytes were examined with the following results.

1. The degree of survival of target cells was inversely related to the number of sensitized lymphocytes. Graphic plots of the data indicated that this relationship was an exponential one similar to "single-hit" inactivation phenomena. One interpretation which could be placed on these results is that a single lymphocyte, if immunologically active, was sufficient to destroy or at least have a detectably adverse effect on one target cell. Furthermore, from such a model it could be computed that, of the lymphocytes derived from an immunized animal, approximately 1 to 2 per cent of the cells were immunologically active; *i.e.*, capable of demonstrable destructive activities against homologous target cells *in vitro*.

2. Morphological studies on the effect of sensitized lymphoid cells on homologous target cells, after various lengths of time in culture, showed that by 7 hours of incubation, the attacking lymphocytes firmly adhered to the target cells. The cytotoxic effect of these lymphocytes generally occurred after the 20th hour. Quantitative studies supported this conclusion; the latent period, *i.e.*, the time required for detectable degrees of target cell destruction to occur, was approximately 20 hours.

3. A consequence of the incubation of target cells with *normal* lymphoid cells or even with small numbers of sensitized lymphoid cells was an *increase* in the rate of division of the target cells. As might be expected, this was reflected in a shorter doubling time of these cells.

4. Extracts prepared from sonically disrupted sensitized lymphocytes proved to be no more deleterious to target cells than similar preparations from normal lymphoid cells. Furthermore, no evidence could be obtained that sensitized lymphoid cells, separated from target cells by a Millipore membrane, were cytocidally effective. These data indicated that if a cell-bound substance is involved in the destruction of homologous cells, either it is not toxic by itself, or it cannot be detached from the sensitized cells. In any case, close apposition of the lymphocytes to the target cells is apparently required for the destruction of the latter *in vitro*.

5. *Serum* obtained from immunized animals, if heat-inactivated, did not adversely affect homologous target cells; if employed fresh, slight degrees of toxicity resulted. Specific isoimmune sera did not impart any detectable degrees of immunological reactivity upon otherwise *normal* lymphoid cells. Immune sera, even in high concentrations, did not augment the effect of sensitized lymphoid cells upon homologous target cells; rather a slight inhibitory effect of these sera was detected.

6. Attempts to detect the presence of complement activity, which might have been provided by the lymphoid cells in culture, were unsuccessful.

On the basis of these results, it was suggested that the destruction of homologous target cells by sensitized lymphoid cells occurs as a two step process. First, the attacking lymphocytes attach to their targets *via* a non-toxic cell-bound substance having an immunologic specificity, and then, destruction of the target cells follows the result of some process dependent on the metabolic activity of the attacking lymphoid cells.

The author wishes to express his sincere appreciation to several of the staff of the Wistar Institute for helpful discussions on various aspects of this study; in particular, Dr. V. Defendi, Dr. J. D. Ross, Dr. L. Manson, Dr. P. Kraemer, Dr. A. Kozinski, and Mr. E. Shahn. The complement-fixation tests were performed by Dr. R. Gilden. It is a pleasure to acknowledge the assistance of Dr. R. E. Billingham in the preparation of this manuscript; Dr. W. K. Silvers for an apparently inexhaustible supply of animals; and especially Mrs. L. Baker and Mrs. S. Schroeder for excellent and patient technical assistance.

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