REJECTION OF ASCITES TUMOR ALLOGRAFTS

I. Isolation, Characterization, and In Vitro Reactivity of Peritoneal Lymphoid Effector Cells from BALB/c Mice Immune to EL4 Leukosis*

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Ascites tumors grow exponentially in the peritoneal cavity of syngeneic mice (1). They also proliferate freely for a number of days in allogeneic mice before they are rejected. The rejection of an ascites tumor by an incompatible host affords a model for the study of homograft reactivity in which vascular and stromal effects are minimal (2). One of the most striking features of the reaction is the sudden transition from exponential growth to cataclysmic decline, occurring as it does within the span of less than 24 hr (3). The cause for this abrupt fall in tumor cell number is not clearly understood. Since antibody-mediated reactions are characteristically rapid, we have previously sought evidence that the rejection of ascites tumors is due to the effects of antibody (4). These attempts have been only partially successful. It is known, however, that peritoneal exudate cells (PEC)[1] tend to accumulate during ascites tumor growth (3), and that host cells present in the ascites fluid have demonstrable effects in both transfer and in vitro test situations (5–7). We have, therefore, attempted to identify the most active subpopulation of cells in the peritoneal exudate and to investigate its cytotoxic effects in vitro. Results of the present investigations suggest that it is possible to isolate from BALB/c mice the population of lymphoid effector cells intimately associated with the rejection of EL4 ascites leukemia.

Materials and Methods

Animals and Immunization.—3–6-month-old mice of the following strains were used: BALB/c Cr, C57BL/Spr, and DBA/2 Ha males (Roswell Park Memorial Institute, West

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[1] *Abbreviations used in this paper:* MEM, minimum essential medium; PBS, phosphate-buffered saline; PBS-FCS, PBS supplemented with 10% heat-inactivated fetal calf serum; PEC, peritoneal exudate cells; RPMI, RPMI 1640 plus 10% heat-inactivated FCS, 100 units/ml penicillin, and 100 µg/ml streptomycin.

Seneca, N. Y.); male BALB/cJ, female A/J and C57BL/6J (Jackson Laboratories, Bar Harbor, Maine). Mice were immunized against allogeneic tumors by a single intraperitoneal injection of 3×10^7 tumor cells suspended in phosphate-buffered saline (PBS)[2] (8). Peritoneal exudate cells or other lymphoid cells were harvested 10–11 days later; the tumor was rejected by the 10th day.

Tumors.—ELA leukemia of C57BL, SaI spindle cell sarcoma of A/J, and L1210 leukemia of DBA/2 were carried as ascites in the appropriate strains.

Media.—Immune lymphoid cells or tumor target cells were collected in PBS supplemented with 10% heat-inactivated fetal calf serum (PBS-FCS), washed, and resuspended in either PBS-FCS or in RPMI 1640 supplemented with 10% heat-inactivated FCS, penicillin, 100 units/ml, and streptomycin, 100 µg/ml (RPMI).

Cell Preparation.—Animals were killed by CO_2 narcosis. Cells were harvested in the cold, and nucleated cells were counted and examined for viability by trypan blue exclusion.

Peritoneal exudate cells (PEC): A midventral skin incision exposed the intact peritoneum. The peritoneal cavity was entered with a 22 gauge needle, and 5 ml of PBS-FCS plus 5 units/ml sodium heparin (Upjohn Co., Kalamazoo, Mich.) were injected. The abdomen was vigorously massaged to effect mixing and the fluid content withdrawn by syringe. The above procedure was repeated once. The recovered suspension was passed through a double thickness of cotton gauze to remove clumps, and the remaining cells were washed with PBS-FCS by centrifugation at 200 g for 10 min.

Nonadherent peritoneal exudate cells (nonadherent PEC): About 50–100 $\times$ 10^6 PEC in 10 ml medium (RPMI or PBS-FCS) were loaded onto a 15 x 120 mm nylon wool column and incubated for 30 min at 37°C (9). The nonadherent cells were eluted with excess medium and washed.

Spleen cells: Spleens were removed and placed in cold PBS-FCS, minced, pressed through a fine nylon mesh, filtered through cotton gauze, and washed twice.

Peyer's patches, thymus, and lymph node cells: Lymph nodes (cervical, axillary, mesenteric, inguinal), thymus, and Peyer's patches were collected into cold PBS-FCS and teased apart with fine forceps. The cell suspensions were allowed to stand to remove large clumps.

Blood leukocytes: Mice were bled from the retroorbital sinus into heparin. The whole blood was diluted 1:4 in PBS, further diluted 1:2 with Plasmagel (Laboratoire Roger Bellon, Neuilly, France), and allowed to sediment at room temperature for 20–40 min. The leukocyte-rich supernatant was removed, spun at 200 g for 10 min, and washed in PBS-FCS.

In Vitro Assessment of Tumor Cell Lysis.—The in vitro lysis of tumor cells was determined by incubating ^{51}Cr-labeled tumor cells with sensitized lymphoid cells and determining the amount of ^{51}Cr released into the medium. The principles of this procedure have been previously described (10–12). Tumor cells to be used as target cells were removed from the peritoneal cavity of syngeneic hosts by syringe, washed in cold PBS-FCS, and adjusted to a concentration of 3×10^7 cells/ml. 1 ml of tumor suspension was incubated with 100 µCi (^{51}Cr) Na_2CrO_4 (Amersham/Searle Corp., Arlington Heights, Ill.) for 45 min at 37°C with occasional shaking. At the end of the labeling period, the cells were washed twice with 45 ml PBS-FCS and recounted. Usually 95–100% of the labeled cells were recovered. 5×10^4 ^{51}Cr-labeled tumor cells were then mixed with varying numbers of lymphoid cells in 1 ml aliquots and plated in 35 mm tissue culture plates (Falcon Plastics, Oxnard, Calif.). The plates were incubated on a Rocker Platform (Bellco Glass, Inc., Vineland, N. J.) at 5 cycles/min at 37°C in an humidified atmosphere of 5% CO_2 in air. In those experiments with PBS-FCS as the sole medium, CO_2 was omitted. At the end of the incubation period, 1 ml cold PBS-FCS was added to each plate and the contents transferred to 12 x 75 mm plastic tubes by suction. The tubes were centrifuged in the cold at 900 g for 10 min and the radioactivity of the supernatants assayed in a well-type gamma scintillation counter (Baird-Atomic Spectrometer, Baird-

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[2] All tissue culture reagents were from Grand Island Biological Co., Grand Island. N. Y.

Atomic, Inc., Cambridge, Mass.). In order to determine the maximum amount of releasable radioactivity, labeled tumor cells were frozen and thawed three times in 0.05 N NaCl, centrifuged, and the radioactivity of the supernatant assayed. The supernatant radioactivity of the frozen and thawed tumor cells represented 90–95% of the total radioactivity incorporated into the labeled cells.

The lysis of tumor cells by immune lymphoid cells was assayed in triplicate. The relative standard error of the mean of released radioactivity seldom exceeded 3%. Variability between repeat experiments on different days was no greater than ±15%. Results are expressed either as the per cent of chromium released or as the degree of lysis, given as a per cent, according to the following equations:

$$\text{Chromium released (as per cent)} = \frac{\text{Counts per minute released by tumor in presence of immune cells}}{\text{Counts per minute released by freeze-thawed tumor cells}} \times 100. \quad (1)$$

Degree of lysis
(as per cent)

$$= \frac{\left(\begin{array}{c}\text{Counts per minute released}\\ \text{by tumor in presence of}\\ \text{immune cells}\end{array}\right) - \left(\begin{array}{c}\text{Counts per minute released}\\ \text{by tumor in absence of}\\ \text{immune cells}\end{array}\right)}{\text{Counts per minute released by freeze-thawed tumor cells}} \times 100. \quad (2)$$

We have shown elsewhere that ^{51}Cr release correlates with trypan blue uptake.[3]

RESULTS

In Vitro Cytolytic Reactivity and Specificity of Different Immune Cell Populations.—BALB/c mice were inoculated intraperitoneally with 3×10^7 EL4 cells. 10 days later, cells from the thymus, lymph nodes, Peyer's patches, spleen, blood, and peritoneal cavity were compared for their cytolytic effect against ^{51}Cr-labeled EL4 cells. Results, summarizing several experiments, are expressed graphically in Fig. 1. It is apparent that, in animals immunized by the intraperitoneal route, the greatest specific activity is given by the PEC. Lymph nodes, Peyer's patch, thymus, and spleen cells were relatively inefficient, and even blood leukocytes fell considerably short of the killing produced by PEC.

In order to see if the high cytolytic reactivity observed for PEC was due to the intraperitoneal route of administration, or if tumor given elsewhere would elicit high reactivity in the PEC, animals were inoculated subcutaneously with tumor. A total of 3×10^7 EL4 cells was injected at multiple sites over the abdominal wall. When the PEC were examined 10 days later, very low cytolytic reactivity was found (Fig. 2). Spleen and peripheral blood leukocytes on the other hand, exhibited reactivity comparable to that found in animals sensitized intraperitoneally. Interestingly, the cell composition of the PEC after subcutaneous immunization differed from normal PEC or PEC obtained after intra-

[3] Sullivan, K., G. Berke, and D. B. Amos. 1972. [51]Cr leakage from and uptake of trypan blue by target cells undergoing cell mediated destruction. *Transplantation.* In press.
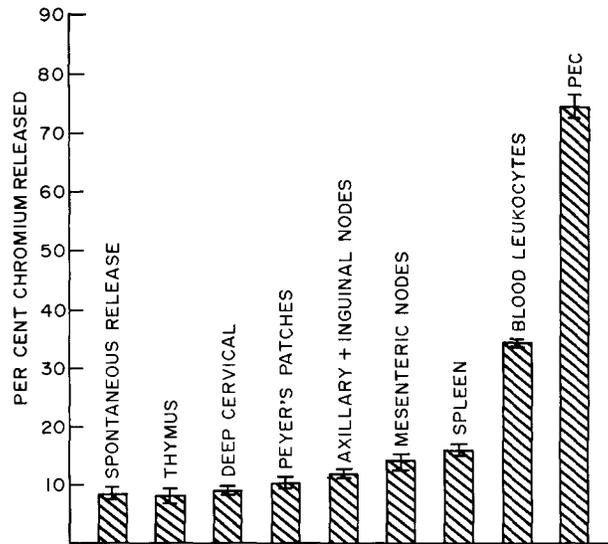
Fig. 1. ^{51}Cr release from labeled EL4 cells incubated with BALB/c cells immune to EL4. Sensitized cells from the indicated sources were mixed with ^{51}Cr-labeled EL4 cells in RPMI at a 10:1 ratio and incubated with rocking for 2.5 hr. Values given are the means of three replicate cultures. Vertical bars indicate ranges.
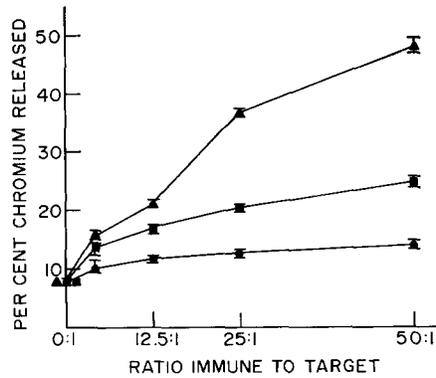

Fig. 2. Cytolytic reactivity of immune lymphoid cells from different sources after subcutaneous injection of EL4. Immune BALB/c spleen cells (■), blood leukocytes (▲), and PEC (●) were mixed with ^{51}Cr-labeled EL4 in PBS-FCS, rocked for 2 hr at 37°C, and the per cent of ^{51}Cr released determined. Values are means of triplicate cultures; vertical bars indicate ranges.

peritoneal injection (see also Table III). 40% of the cells recovered were large pyroninophilic lymphoid cells; 50% were small- to medium-sized lymphocytes, and the few remaining cells were mast cells, polymorphonuclear leukocytes, or were unidentified. Only a few macrophages were observed in more than 5000

cells examined. The total number of PEC recovered per mouse was approximately 7×10^6, as compared with 2-3×10^6 for normal BALB/c mice. It was concluded, therefore, that the high cytolytic reactivity of the PEC after intraperitoneal inoculation (Fig. 1) was caused by localization of effector cells at the site of tumor rejection.

Experiments were designed to see if the cytolytic reaction was specific or if chromium would also be released from third-party or bystander cells. Spleen cells from BALB/c immunized intraperitoneally against EL4 were preincubated with unlabeled EL4 or SaI cells for 2 hr, after which labeled SaI or EL4 cells

TABLE I

Specificity of Target Cell Destruction In Vitro

Preincubation target*	Indicator target	Degree of lysis		
		BALB/c anti-EL4 spleen	C57BL/6J anti-SaI spleen	BALB/c anti-EL4 PEC
		%	%	%‡
EL4	EL4§	49.6‖	0.1‖	—
EL4	SaI§	0	27.4	—
SaI	EL4§	46.1	0.3	—
SaI	SaI§	0	26.4	—
—	EL4 + SaI§	0.4	29.1	0.2
—	SaI + EL4§	46.5	0	75.8
—	EL4§	48.3	0	83.1
—	SaI§	0.3	29.5	2.4
—	EL4 + EL4§	—	—	77.0
—	L1210§	—	—	0

* 50×10^3 tumor cells and 5×10^6 spleen cells were preincubated for 2 hr in 1 ml aliquots before 50×10^3 indicator cells were added. Where preincubation was not done, 5×10^6 spleen cells were added to 50×10^3 of each indicator target cell.

‡ The ratio of PEC to indicator cells was 20:1 and the degree of lysis was determined after 2.5 hr of incubation.

§ ^{51}Cr-labeled tumor cells.

‖ Degree of lysis was determined after 4 hr incubation; values given are means of triplicates.

were added. While there was no release of label from SaI, there was good release from EL4 (Table I). In another series, spleen cells from C57BL/6 mice, immune to SaI and preincubated with unlabeled SaI or EL4, released label from subsequently added SaI but not from EL4. When both EL4 and SaI cells were added simultaneously with only one cell type labeled, specific lysis was again observed. In each case, the cytolytic capacity of the immune cells was demonstrated by their ability to release label from the appropriate target. Preincubation made the nonspecific participation of soluble substances formed by the killer cells unlikely under the conditions of these experiments (13, 11). Comparable results (Table I) were obtained if PEC were substituted for spleen cells, or if L1210 was substituted for SaI.

Adherent vs. Nonadherent PEC in the In Vitro Destruction of Tumor Cells.— Crude PEC suspensions obtained from BALB/c immunized intraperitoneally against EL4 contained macrophages and lymphocytes. Since PEC were so efficient in target cell destruction (Fig. 1), the reactivity of the two constituent cell types was compared. Aliquots containing 1×10^6 PEC in 1 ml RPMI were placed in 35 mm polystyrene Petri dishes and rocked at 37°C for 90 min. The medium phase was removed to provide nonadherent cells, and the plates were washed twice with medium to remove any further nonadherent cells. Other plates were rocked and the cells left undisturbed. Finally, nonadherent cells were added back to some of the washed plates to give a reconstituted PEC suspension. Concomitant experiments, designed to test whether the release of conditioning substances into the medium affected the behavior of the separated

TABLE II
Cytolytic Activity of Adherent and Nonadherent BALB/c Anti-EL4 PEC

Cells	Medium*	Degree of lysis (hr)	
		1	2
		%	%
Undisturbed	Old	50.7‡	76.8
Nonadherent	Old	47.0	64.7
	Fresh	42.1	68.1
Adherent	Old	7.8	20.3
	Fresh	9.8	21.3
Reconstituted	Old	52.3	73.8
	Fresh	51.6	76.3

* See text.
‡ Values given are means of triplicates.

subpopulations, were also performed. Medium removed with nonadherent cells was saved. The cells were then brought to their aliquot concentration either in this (old) medium or in fresh medium and examined for their lytic activity against ^{51}Cr-labeled EL4 cells. The results are expressed in Table II. Clearly, most of the activity resided in the nonadherent population. Although there were fewer nonadherent cells, the degree of lysis approached that obtained in the undisturbed or reconstituted plates, while the level of killing by the adherent cells was correspondingly low. The relatively inefficient lysis by the adherent population was not affected by restoration of the original medium, nor was the effectiveness of the nonadherent cells influenced by changing the medium.

Quantitative Aspects of In Vitro Cytotoxicity by Nonadherent PEC.—The preceding experiments indicated that cells nonadherent to polystyrene were effective in target cell lysis. In order to obtain a better separation, nonadherent PEC from immunized mice were eluted from nylon wool columns. Microscopic examination revealed that less than 4% of the eluted cells had macrophage-like

characteristics as determined by morphology and by yeast particle ingestion. The vast majority of cells were small- to medium-sized lymphocytes; the remaining cells were large lymphoid, polymorphonuclear, mast, or unidentified (Table III). Although only two mitotic figures were seen in over 5000 cells examined, data given in Table III demonstrate increased thymidine-^{3}H incorporation.

A detailed kinetic analysis of target cell destruction provided information on the rate of killing. Nonadherent immune PEC were serially diluted and mixed with 5×10^4 ^{51}Cr-labeled EL4 cells. The mixtures were plated and rocked and the per cent of ^{51}Cr released determined at various time intervals. The results

TABLE III

BALB/c PEC Morphology and Nucleotide Incorporation

Cells	Cell type*					Incorporation‡	
	Lymphoid		Histiocytes	Polymorpho-nuclear	Other	Uridine-^{3}H	Thymi-dine-^{3}H
	Small-medium	Large					
	%	%	%	%	%	*cpm*	*cpm*
Immune crude PEC	38	<1	47	5	9	—	—
Immune nonadherent PEC	91	<2	2	2	3	1350	11,170
Normal PEC	60	3	31	3	4	2500	2040

* Differentials were done on cover slip films stained with Wright's or Giemsa. Total number of cells examined = 5073.

‡ 1 μCi of uridine-^{3}H (20 Ci/mM) or thymidine-^{3}H (3 Ci/mM)[4] was added to 1×10^6 cells, plated, and rocked for 2 hr at 37°C. After incubation, the cells were washed three times with PBS, solubilized with NCS solubilizer,[5] and counted (toluene base scintillation solution) in a Packard Tri-Carb (Packard Instrument Co., Downers Grove, Ill.). 96% of the incorporated radioactivity was found to be in acid (10% cold trichloroacetic acid)-insoluble material.

(Fig. 3) indicate that lysis reached a maximum rate very shortly after mixing,[6] and was strictly dependent on the killer-to-target cell ratio. The time taken to reach completion was a function of the number of killer cells.

Using a similar kinetic approach, target cell destruction by nonadherent PEC was compared with the destruction produced by crude PEC and spleen cells. The data, expressed both as per cent chromium released per hour and as a function of the ratio of immune cells to target cells, are given in Table IV. From these results, the high efficiency of nonadherent PEC in killing is established on a per cell basis. At a 5:1 immune-to-target cell ratio, values of 48, 22, and

[4] Schwarz BioResearch Inc., Orangeburg, N. Y.

[5] Amersham/Searle Corp.

[6] With the present technique employed for the determination of ^{51}Cr release, time measurements shorter than 45 min are impractical.
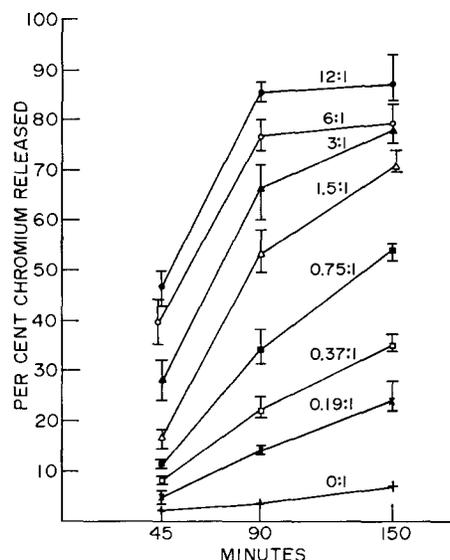
FIG. 3. Time-course of chromium release from EL4 cells incubated with nonadherent PEC from immune BALB/c mice. Values given are means of triplicate cultures; vertical bars indicate ranges. Numbers indicate killer-to-target cell ratios.

TABLE IV

Rate of Cytolysis of ^{51}Cr-labeled EL4 by BALB/c Anti-EL4 Immune Cells

Immune:target ratio	Chromium release*		
	Nonadherent PEC	Crude PEC	Spleen
	%/hr	%/hr	%/hr
0:1	2.7	3.3	3.0
1:1	23.1	10.0	3.2
5:1	48.4	22.5	5.1
10:1	60.0	30.0	7.8
25:1	—	42.7	9.5
50:1	—	44.7	17.5
100:1	—	—	30.2

* Chromium release was determined after 1 hr incubation; the given values are means of triplicates.

5% label released per hour were obtained with nonadherent PEC, crude PEC, and spleen cells respectively.

The Requirement for Serum in the Cytolytic Reaction.—In this system, significant killing can be obtained in a very short time; because of this, it was possible to examine components required in the medium. Examination of various types of media, including Eagle's minimal essential medium (MEM), RPMI 1640, Medium-199, Dulbecco's modified MEM, Hanks' balanced salt solution, and

PBS, have shown that optimal lysis can be obtained with a simple medium, provided it is supplemented with heat-inactivated fetal calf serum. Additional experiments using PBS supplemented with human, horse, mule, rabbit, sheep, and rat serum gave results similar to those obtained with fetal calf serum (Table V). Guinea pig serum and sera from several mouse strains were less supportive. Since complex media also require serum to support the killing reaction, it seemed possible that the limiting factor for lysis was in the added serum. The extent of lysis of EL4 by BALB/c anti-EL4 nonadherent PEC was, therefore, determined as a function of the concentration of serum and other proteins. Results of seven separate experiments (Fig. 4) demonstrate that the cytolytic reaction was indeed dependent on serum concentration. Similar results were obtained when FCS, extensively dialyzed against PBS, was used instead of whole serum. Bovine serum albumin, human transferrin, bovine gamma globulin, human albumin, ovalbumin, and sheep gamma globulin did not support the cytolytic

TABLE V

Effect of Sera from Various Sources on the Cytolytic Reaction

| | Serum* | | | | | | | | Mouse | | | |
	FCS	Human	Rat	Rab-bit	Sheep	Mule	Agamma-horse	Guinea pig	BALB/c	C3H/f	C57/f	C57BL/Spr
Per cent ^{51}Cr released‡	73.9	82.7	66.3	74.9	80.9	74.0	62.0	47.3	45.3	38.0	60.4	56.0

* Heated at 56°C for 1 hr and used at 10% in PBS.

‡ ^{51}Cr released was determined after 60 min of incubation at a 2:1 ratio of immune nonadherent PEC to EL4 target.

reaction. To rule out the possibility that the fetal calf serum was contributing heat-labile components, the serum was heated at 56°C for 1 hr immediately before use. There was no loss of activity. The addition of carrageenin or of cobra venom factor also failed to reduce the extent of killing.

Since the killing activity was highly dependent upon serum concentration, experiments to see if the essential serum component was consumed during cytotoxic reactivity were carried out. BALB/c anti-EL4 nonadherent PEC were rocked at 37°C with unlabeled EL4 cells in PBS containing limiting concentrations of heat-inactivated FCS. At the end of the incubation, the suspensions were collected, centrifuged, and the media saved. Controls were prepared in the same way from media that had been incubated with EL4 cells, nonadherent PEC, or crude PEC alone, or which had been incubated without added cells (medium control). The media from the various groups were serially diluted and examined for their capacity to support the killing of ^{51}Cr-labeled EL4 cells by a fresh preparation of immune nonadherent PEC. The results (Fig. 5), summarizing three separate experiments, showed that medium which had previously
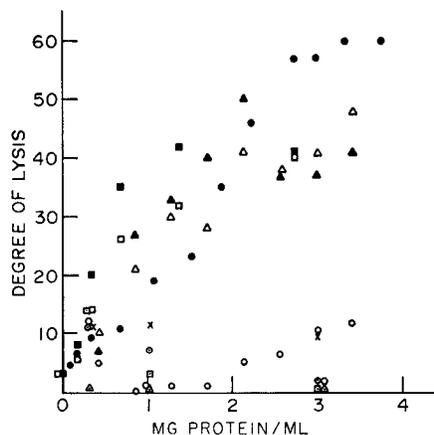
FIG. 4. Cytolysis as a function of FCS and purified protein concentrations. The solid symbols, (●, ■, ▲) represent heat-inactivated fetal calf serum in PBS at the indicated concentrations with killer-to-target cell ratios of 3:1, 8.5:1, and 2:1. Plates were rocked at 37°C for 60, 60, and 90 min respectively. The open symbols, (□, △) represent heat-inactivated dialyzed fetal calf serum and conditions corresponding to the appropriate filled symbol. When purified proteins (○, human transferrin, Sigma Chemical Co., St. Louis, Mo.; ▣, bovine gamma globulin, Pentex Biochemical, Kankakee, Ill.; △, human albumin, Pentex; hexagons, sheep gamma globulin, Pentex; ○, BSA, Pentex; and X, ovalbumin 2X, Worthington Biochemical Corp., Freehold, N. J.) were used, the killer-to-target cell ratio was 2:1 and the degree of lysis was determined after 90 min at 37°C rocking. All cells were washed thoroughly and added to the reaction mixture in PBS. Each point given is the mean of triplicate cultures.
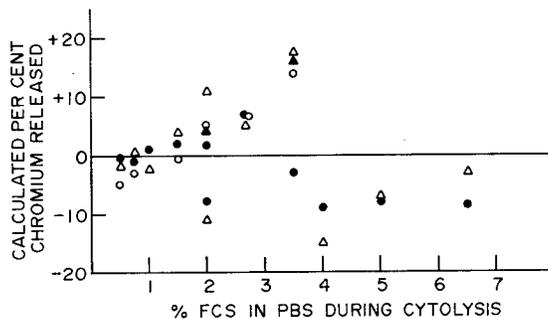

FIG. 5. Consumption of medium components during the cytolytic reaction of BALB/c anti-EL4 nonadherent PEC against EL4 tumor cells. The results are expressed as the per cent of chromium release obtained with the various media minus the per cent of chromium release obtained with media preincubated without cells plotted against the concentration of FCS in PBS employed during the subsequent cytolytic reaction. PBS containing 8, 4, or 3% FCS was preincubated with EL4 (●), immune nonadherent PEC (○), immune nonadherent PEC plus EL4 (△), or immune crude PEC (▲) for 60–120 min at 37°C with rocking. Killer-to-target cell ratios were 10:1–1:2.

supported cytotoxicity remained supportive even when the included serum was at very low concentration. In this figure, values above the horizontal line represent increased lysis after incubation with effector cells and target; values below the line show decreased efficiency. The random scatter and the over-all closeness to the control value show there was neither increased nor decreased activity.

Effects of Trypsin Treatment, Freeze-Thawing, and Homogenization of PEC on Their Killing Activity.—The experiments to this point have shown that the

TABLE VI
Effect of Trypsin Treatment on Killing Reactivity

Experi-ment	Cells	Serum*	Chromium release (min)‡				
			45	60	90	120	150
					%		
1	Crude immune PEC§	IMS	3.8		10.0		24.0
		NMS	3.7		14.5		38.1
	Normal PEC§	IMS	2.6		5.2		7.5
		NMS	2.0		3.5		6.2
	Crude immune PEC	FCS	35.9		73.9		95.2
	Normal PEC	FCS	3.2		3.9		6.2
2	Nonadherent immune PEC§	IMS		11.3		27.2	
		NMS		12.0		23.7	
		FCS		11.0		27.2	
	Nonadherent immune PEC	IMS		38.8		70.4	
		NMS		32.6		58.8	
		FCS		41.9		71.3	

* Cells were incubated after treatment with either trypsin or PBS-FCS for 30 min at 37°C in immune BALB/c anti-EL4 serum with a cytotoxic titer against EL4 of 1/512 (IMS), normal BALB/c serum (NMS), or FCS; all sera were diluted 1:2 in PBS.

‡ PEC to target cell ratio 10:1. Values are means of triplicates.

§ Trypsin-treated cells. Experiment 1: 5×10^6 normal or immune crude PEC incubated for 30 min at 37°C in a 5 mg/ml trypsin (Worthington) solution in PBS. Experiment 2: 12×10^6 nonadherent PEC incubated for 30 min at 37°C in a 3 mg/ml trypsin solution in PBS. All cells were washed three times with PBS-FCS after trypsin treatment. Nontrypsin-treated cells were incubated in PBS-FCS.

killing effect is specific, potent, largely due to the nonadherent cells, and dependent on the concentration of FCS. Experiments were undertaken to investigate whether killing was related to the presence of a trypsin-sensitive cell-bound antibody (7). Nonadherent PEC or crude PEC were treated with trypsin, washed, and left to recover in the presence of a hyperimmune BALB/c anti-EL4 serum, normal BALB/c serum, or fetal calf serum. At the end of the recovery period, the cells were washed and their ability to damage target cells was measured. The results of these experiments (Table VI) showed that PEC, either nonadherent or crude, when treated with trypsin, temporarily lost much

of their cytolytic power (14) although 95 % of the treated cells excluded trypan blue. Activity returned with time; the regeneration of activity occurred in the presence of FCS just as well as it did in immune or in normal BALB/c serum. These experiments do not support the notion that the high reactivity found in this system is due to a trypsin-sensitive, cell-bound antibody similar to that described for macrophage-mediated cytolysis (7).

Experiments were performed to determine whether a cell-free lysate made from killer cells, or medium obtained from a culture where lysis had occurred, would affect ^{51}Cr-labeled target cells. A suspension of nonadherent BALB/c anti-EL4 PEC was divided into aliquots. The first was frozen and thawed three

TABLE VII

Cytolytic Effect on ^{51}Cr-labeled EL4 of Cell-Free Preparations Made from Nonadherent PEC

Group	Relative concentration	Degree of lysis
		%
Freeze-thaw	1:2*	0.5‡
Homogenate	1:2*	0.0
Medium	1:1§	0.7
	1:2	1.2
	1:4	0.6
PEC	5:1‖	92.2
	1:1	33.7
	0.5:1	17.5

* Preparations were made from 15 × 10⁶ PEC in 2 ml PBS-FCS and diluted in PBS-FCS.

‡ Degree of lysis was determined after 90 min of incubation from triplicate cultures.

§ Dilution in PBS-FCS of medium (PBS-FCS) collected from a cytolytic culture incubated for 90 min with rocking which had a killer-to-target cell ratio of 10:1.

‖ Values refer to killer-to-target cell ratio.

times; the second was homogenized by 20 strokes in a Teflon-glass homogenizer; the third was preincubated with unlabeled EL4 cells. The supernatants were examined for their effects on EL4 cells. There was no evidence of a cell-free constituent capable of destroying target cells (Table VII). Control cells derived from the same starting material and held on ice were fully active.

DISCUSSION

The identity of the specific cell type(s) responsible for cell-mediated graft rejection is an unsettled question. However, specifically sensitized lymphoid cells have been shown to function in the immunological rejection of homografts (15) and to mediate, in vitro, a direct cytotoxic reaction upon target cells genetically similar to the tissue used for immunization (16, 17). Thoracic

duct lymphocytes injected into histoincompatible recipients soon develop into large pyroninophilic cells in the afferent phase of cellular responsiveness. These cells undergo mitosis yielding small- and medium-sized lymphocytes (18). Ginsburg (19) and Denham et al. (20) have implicated a large lymphoid cell as the effector of cell-mediated immunity, while Weiner's electron microscope studies (21) have associated a ribosome-rich small lymphocyte with damage to grafted tissue.

Effector cells used in cytotoxicity tests are generally derived from spleen or lymph nodes of immune animals. Such conventional lymphoid cell preparations undoubtedly contain a large number of irrelevant cells. Attempts to obtain a specifically immune effector cell population by using draining lymph nodes (22) or cellular immunoabsorbents (23, 24) have been only partially successful. Ideally, one would like to study those effector cells anatomically associated with a rejected graft. While this is technically difficult with solid grafts, the use of an allotransplanted ascites tumor provides, in readily accessible suspension, a unique population of effector cells capable of causing a vigorous destruction of appropriate target cells in vitro. This source of effector cells has received surprisingly little attention. Early experiments with PEC concentrated on macrophages (6, 7). Recently, however, Takasugi and Klein (25) have described the efficacy of crude PEC in target cell destruction. Oppenheim et al. (26) have shown that PEC, induced by mineral oil, from guinea pigs sensitized by intradermal inoculations depressed the uptake of tritiated thymidine by tumor cells.

We have found that shortly after the disappearance of EL4 ascites tumor from BALB/c mice a population of nonadherent PEC appears which is uniquely potent in the in vitro destruction of EL4 cells. These cells provide an avenue for the characterization of at least one type of effector cell and may, because of their unusual potency, allow more complete studies on the mechanism of cell-mediated allograft destruction. The cells appear to be almost exclusively small- to medium-sized lymphocytes. They are intensely pyroninophilic and, with Wright's stain, have densely basophilic nuclei with pale blue rims of cytoplasm. Examination of over 5000 cells revealed no more than two mitotic figures. It should be noted, however, that this population of effector cells is capable of a fivefold increase in the incorporation of tritiated thymidine as compared with normal PEC. The role of the macrophage as the major effector cell in this system could be excluded. The efficiency of killing, on a per cell basis, was increased rather than diminished by removal of the macrophages. The effector cells differed in their properties from activated macrophages by regaining activity on incubation with normal as well as with immune serum after trypsin treatment (7). It is our belief that the cell active in this system is a particular kind of small- or medium-sized lymphoid cell and that the blastoid phase of lymphocyte activation (18, 27) involves the priming and programming of these cells.

A quantitative interrelationship between the effector cells and the target cells has been established, and various parameters affecting target cell destruction

have been elucidated. We have shown that the rate of target cell destruction is dependent on the concentration of effector cells. It was also found to be dependent on the concentration of target cells.[7] The unexpectedly high reactivity of nonadherent PEC is best documented in those experiments where the rate of target cell destruction, i. e. per cent chromium released per hour, was measured as a function of killer-to-target cell ratio. When the rates were compared for column-separated, nonadherent PEC, crude PEC, and spleen cells at a killer-to-target cell ratio of 10:1, values of 60, 30, and 8% ^{51}Cr released/hr, respectively, were obtained. The rates of EL4 destruction by nonadherent PEC reported here are far greater than those reported where spleen or lymph node cells were employed (28–30, 11). It seems most likely that the high efficacy of nonadherent PEC is due to the presence of a high proportion of effector cells at the reaction site. However, we are aware of a different explanation, namely, that the content of effector cells in PEC is similar to that of spleen or lymph nodes but that the mechanism of lysis produced by them is different. This possibility seems unlikely. Particular features of target cell destruction by spleen and other lymphoid cells are shared by PEC. The findings that the reaction is specific and is produced by viable sensitized lymphoid cells, their sensitivity to trypsin and subsequent recovery in normal serum, together with the demonstration that target cell lysis is initiated shortly after mixing and proceeds with a rate dependent on both effector and target cell concentration, are examples of the similarity (28–30, 11). We conclude that the effects of PEC and those of lymphoid cells from other sources are qualitatively similar but quantitatively different.

The dependence of the cytolytic reaction on serum concentration and the observation that optimal reactivity can be obtained in phosphate-buffered saline supplemented with serum of any of a variety of species suggests that a serum component(s) is engaged in the reaction. Bovine serum albumin or human transferrin, as well as several other purified proteins, could not replace serum, nor could the combination of FCS dialyzate and bovine serum albumin. Heated serum (56°C for 1 hr) was as good as unheated serum, and serum extensively dialyzed against PBS was fully supportive. It is interesting that the serum component(s) is not exhausted by the reaction, suggesting a possible catalytic function.

The poor cytolytic activity of peritoneal cells obtained, without the administration of irritants, from animals injected subcutaneously with EL4 supports our concept of effector cell accumulation at the site of allograft rejection. These PEC produced little tumor cell lysis even at a 50-fold ratio of PEC to target cells. Target cell destruction by spleen and blood leukocytes from these same animals was as expected. These experiments offer an interesting contrast to

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[7] Berke, G., K. A. Sullivan, and D. B. Amos. Rejection of ascites tumor allografts. II. A pathway of cell-mediated destruction in vitro by peritoneal exudate effector cells. Manuscript in preparation.

those in which lysis was obtained at less than one PEC per target cell when the tumor had been rejected in the peritoneal cavity.

SUMMARY

Peritoneal exudate cells (PEC), obtained after the rejection of EL4 leukemia by BALB/c mice, are much more effective in the specific in vitro destruction of ^{51}Cr-labeled EL4 cells than are spleen, thymus, lymph node, or peripheral blood lymphocytes. The presence of a large number of effector cells at the site of graft rejection is reflected in the potent cytolytic activity seen in vitro. Effector cells temporarily lose cytolytic reactivity when treated with trypsin but regain reactivity with time. This recovery occurs in normal as well as in immune serum. The destructive reactivity of PEC is increased when macrophages are removed. The remaining population of nonadherent PEC is composed primarily of small- to medium-sized lymphocytes. Complex tissue culture media are not needed, but there is a definite requirement for serum. The required serum component is heat stable, nondialyzable, and is not consumed during the reaction. The use of an ascites allograft system made these observations possible and permitted the isolation of those host cells intimately associated with rejection.

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