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DETECTION OF CYTOTOXIC T LYMPHOCYTES IN THE EL4 MOUSE LEUKEMIA SYSTEM: INCREASED ACTIVITY OF IMMUNE SPLEEN AND PERITONEAL CELLS FOLLOWING PREINCUBATION AND CELL FRACTIONATION PROCEDURES[1]

by

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The cytotoxic activity of spleen and peritoneal cells from C57Bl/6 mice immunized with irradiated syngeneic EL4 leukemia cells was tested in vitro *on* ^{51}Cr *labelled target cells following various fractionation and purification procedures. At the peak of the response, immune spleen cells showed very low cytotoxic activities. Fractionation on a simple BSA density gradient yielded a low-density (less than 1.08 g/cm³) spleen-cell population with a five- to 10-fold increased cytotoxic activity. A similar increase was observed after preincubation of immune spleen cells for 24 h at 37° C, confirming results of Ortiz de Landazuri and Herberman (1972). A successive application of preincubation followed by BSA gradient separation resulted in a cumulative 30- to 100-fold increase in cytotoxicity. Removal of high-density small lymphocytes before preincubation led to populations with considerably decreased activity, suggesting the formation of low-density cytotoxic lymphocytes from high-density precursors during preincubation. Immune peritoneal cells showed a three- to five-fold increase in cytotoxicity after (1) removal of phagocytic cells, (2) 24 h preincubation followed by removal of adherent cells, and (3) removal of phagocytic cells followed by preincubation. This indicated that adherent cells were not involved in but appeared to inhibit the cytotoxic activity of immune peritoneal cells. Treatment of preincubated or gradient-separated immune spleen and peritoneal cells with anti-θ serum and complement and passage through Sephadex Ig-anti-Ig columns demonstrated the T-cell nature of the cytotoxic effector cells.* In vitro *studies also showed an enhanced protective effect of pretreated immune spleen cells when compared to the original population.*

Lymphocytes from tumor-bearing hosts are often cytotoxic for autologous tumor cells *in vitro*. Depending on the immune status of the host and/or the assay technique employed, cytotoxicity may reflect killing by cytotoxic T lymphocytes (CTL), antibody-dependent killing by normal non-T cells, or killing by armed macrophages (reviewed by Cerottini and Brunner, 1974). In weak syngeneic tumor systems, cytotoxicity mediated by CTL is often difficult to detect and transient. This may reflect the relatively low frequency of effector cells and/or the presence of factors interfering with the lytic mechanism.

Recent studies have shown that cytotoxic T lymphocytes can be separated and identified on the basis of various physico-chemical properties. In the early phase of the immune response, CTL are predominantly large cells (MacDonald *et al.*, 1973; Greenberg, 1973) of low or medium density (Pelet *et al.*, 1971; Shortman *et al.*, 1972).

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[1] Abbreviations used in this paper: T, thymus-derived (cells); CTL, cytotoxic T lymphocytes; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum.

Their separation from dense, small lymphocytes can therefore be achieved by simple physical methods. In addition, CTL can be separated from macrophages or antibody-dependent effector cells by purification procedures based on adherence, treatment with carbonyl iron, or passage through immunoglobulin (Ig)-anti-Ig columns (Wigzell and Andersson, 1971). With all these procedures, removal of certain cell populations is accompanied by an increased cytotoxic activity of the residual cells as a result of the increase in relative frequency of CTL. Preincubation of immune lymphoid cells (Ortiz de Landazuri and Herberman, 1972) or enzyme treatment (Kedar *et al.*, 1974) may also lead to increased cytotoxicity. In the present study, on which preliminary data have been reported elsewhere (Vasudevan *et al.*, 1973), various procedures were applied, individually and in combination, in order to select for and identify the effector cells formed in C57Bl/6 mice immunized with syngeneic irradiated EL4 lymphoma cells. It will be shown that preincubation and/or density gradient separation of immune spleen cells resulted in populations with considerably increased activity *in vitro* and *in vivo*. A similar enrichment in cytotoxic activity was achieved by removal of adherent and/or phagocytic cells from immune peritoneal cell populations. Cytotoxicity of the processed cells was specific and predominantly mediated by T lymphocytes.

MATERIAL AND METHODS

Animals

Three- to 4-month-old C57Bl/6 (B6) male mice were obtained from our own breeding colony.

Tumor cells

EL4 (C57Bl) leukemia cells, originally induced by dimethylbenzanthracene (Gorer, 1950), and serially transplanted intraperitoneally (IP) in B6 mice in ascitic form, were used for immunization. EL4 cells kept in suspension culture in Dulbecco's modified Eagle's medium (DMEM) containing 10% trypticase broth and 10% fetal bovine serum (FBS) were used as target cells for the *in vitro* cytotoxic assay.

Immunization schedule

Following the schedule of Martin *et al.* (1971), B6 mice were injected IP with 0.5×10^6 irradiated (3,000 R) EL4 cells. A second IP injection of 50×10^6 irradiated (3,000 R) EL4 cells was given 3 weeks later. Each test group consisted of five or six animals.

Preparation of spleen and peritoneal cells

Spleens of immunized or normal control B6 mice were removed aseptically and homogenized by hand in a glass Ten-Broek grinder with DMEM. The cell suspensions were immediately centrifuged for 5 min at $400 \times g$, the sediment resuspended and left standing for 45 min at 4° C. The sediment was discarded and the supernatant cells washed twice and resuspended in DMEM-10% FBS. The number of viable cells was determined by hemocytometer counts using the trypan-blue exclusion test. Peritoneal cells (PC) were obtained by washing the peritoneal cavity with 5 ml DMEM.

Carbonyl iron treatment

To 100×10^6 PC in 20 ml DMEM-15% FBS, 250 mg of sterile carbonyl iron (particle size: 3-5 μ) were added and the cells incubated for 45 min at 37° C. The iron particles were then removed by the addition of a magnetic stirring bar. The efficiency of removal of macrophages was tested by placing the remaining cells in culture; less than 1% macrophage-like cells were observed after 24-48 h.

Density gradient separation

Following the method described by Shortman *et al.* (1972), 100×10^6 spleen cells in 0.1 ml DMEM were dispersed at 4° C in 1 ml of bovine serum albumin (BSA) solution. The latter was previously adjusted to a density of 1.08 g/cm^3 and to pH 7.2, using balanced salt solution isosmotic with mouse serum, (308 mOsmol, equivalent to 0.168 M NaCl). Then 1 ml of the dense BSA solution was layered underneath and 1 ml of diluted BSA solution (final BSA concentration approximately 10%) was layered on top of the cell suspension. The tube was centrifuged at $3{,}000 \times g$ for 10 min in the cold. Red blood cells, cell debris and the high-density lymphocytes were sedimented in the pellet. The cells present in the middle layer of the gradient were removed with a pipette and washed twice, and viable cell counts were determined. Under these conditions, low-density lymphocytes accounted for approximately 15-20% of the original lymphocyte count.

Anti-theta serum treatment

The techniques used for the preparation of and the treatment with AKR anti-θ C3H serum have been described previously (Cerottini *et al.*, 1970). For treatment, 100×10^6 lymphoid cells in 1.6 ml DMEM were mixed with 0.2 ml anti-θ serum and 0.2 ml agar-absorbed rabbit serum diluted 1:2 as a source of complement, incubated at 37° C for 45 min and then washed twice. Normal AKR serum and complement were used in control mixtures. Viable cell recovery after treatment with anti-θ serum and complement was generally 50% as compared to 85% of the original lymphocyte count after treatment with normal serum.

Separation on Ig-anti-Ig column

Immunoadsorption columns were prepared as described by Schlossman and Hudson (1973) with the following modifications. One gram of activated Sephadex G200 was mixed with 20 mg normal mouse immunoglobulin and kept shaking overnight at room temperature. It was then washed with phosphate-buffered saline, and finally washed with DMEM containing 10 mM HEPES and 5% FBS. This gel (8 ml) was then packed into a 10 ml syringe at 4° C and 8 ml of rabbit anti-mouse immunoglobulin was added, incubated for 30 min and then washed with DMEM containing 10 mM HEPES, 20% FBS and 5 mM EDTA (pH 7.2). (As a control, another column was similarly prepared with normal rabbit serum). A suspension of 15×10^6 spleen cells from immunized mice was then applied in 1.5 ml of the above-mentioned EDTA medium, and eluted with 20 ml of the same medium at a flow rate of 2 ml/min. Cells were then thoroughly washed to remove the EDTA. Viability was determined by trypan-blue exclusion, and the recovered cells were assayed for their cytotoxic activity against target EL4 cells. In parallel, eluted cells were also tested for their surface immunoglobulins by immunofluorescence, using fluorescein-conjugated rabbit anti-mouse Ig.

Cytotoxicity test

A slight modification of the procedure previously described in detail (Brunner *et al.*, 1968; Cerottini and Brunner, 1971) was employed. Briefly, 2×10^6 target cells in 0.5 ml Tris-buffered saline were incubated with 0.2 ml (approximately 200 μC) of ^{51}Cr-sodium chromate at 37° C for 30 min. After two washings, the cells were further incubated for 2 h at 37° C. Immediately before the test, the cells were centrifuged and resuspended at 10^5 cells/ml in DMEM 10% FBS. For the test, 0.2 ml of the labelled target cells were added to 0.2 ml of the lymphoid cell suspension in round-bottomed (10×60 mm plastic tubes at varying lymphoid:target-cell ratios, usually at 100:1, 30:1 and 10:1. For each group, duplicate or triplicate tubes were incorporated. The reaction mixtures were gassed with 5% CO_2 in air and then incubated for 20 h in a 37° C shaking water bath (80 cycles/min). After incubation, 0.6 ml phosphate-buffered saline was added to each tube, the cells were centrifuged at $400 \times g$ for 5 min, and 0.5 ml of the supernatant was removed for measurement of radioactivity. The percentage of specific ^{51}Cr release was calculated as:

$$\frac{\text{^{51}Cr release in the presence of immune lymphoid cells} - \text{^{51}Cr release in the presence of normal lymphoid cells}}{\text{maximum releasable ^{51}Cr} - \text{^{51}Cr release in the presence of normal lymphoid cells}} \times 100$$

Spontaneous release of chromium from EL4 cells was 20-30% for the 20 h incubation period. Maximum releasable ^{51}Cr was determined after three cycles of freezing and thawing of labelled target cells. The cytotoxic activity of different cell populations was compared by graphical determination of the number of lymphocytes needed to produce similar amounts of specific ^{51}Cr release (Cerottini and Brunner, 1971).

In vivo *protection test*

Lymphoid cells from immune or normal animals were mixed at different ratios with viable EL4 cells and injected subcutaneously into groups of syngeneic normal B6 mice, each group consisting of five animals. The rate of growth of solid tumors in each animal was noted against time.

RESULTS

Cytotoxicity of immune spleen cells

B6 mice were injected intraperitoneally with 0.5×10^6 heavily irradiated (3,000 R) EL4 cells followed 3 weeks later by 50×10^6 irradiated EL4 cells. On different days after the second injection,

the spleen cells were tested for cytotoxicity against ^{51}Cr-labelled EL4 target cells at various lymphocyte:target-cell ratios, using a 20 h assay. Low but detectable cytotoxic activity was observed on day 7 after the second injection, whereas spleen cells collected either on day 5 or day 9 were less active. The results obtained with day 7 spleen cells in 11 experiments are shown in Table I. It can be seen that the mean value of specific ^{51}Cr release reached 10% (range 8-15%) at a lymphocyte:target-cell ratio of 100:1.

BSA gradient purification. In previous experiments it was shown that the cytotoxic lymphocytes formed early during the response to alloantigens had a buoyant density lower than that of the bulk of small lymphocytes (Pelet *et al.*, 1971; Shortman *et al.*, 1972). It was thus possible to obtain populations enriched in CTL by removing high-density small lymphocytes and selecting the lymphoid cells of a relatively low density.

In order to evaluate this procedure in the syngeneic system under investigation, spleen cells of mice collected 7 days after the second injection of irradiated EL4 cells were separated on a simple BSA density gradient into two fractions. The cells having a density of less than 1.08 g/cm^3 were selected and tested for cytotoxicity. The average yield of the low-density fraction was 15-20%; the high-density fraction contained all remaining cells comprising mainly small and some medium-sized lymphocytes, erythrocytes and cell debris. As shown in four experiments presented in Table I (exp. 4-7), the low-density fraction regularly showed a higher cytotoxic activity than the original population. In experiment 7 (Table I) presented in detail in Figure 1, the purified cells produced 14% specific ^{51}Cr release, at a lymphocyte:target-cell ratio of 10:1, whereas a ratio of 100:1 was required for untreated spleen cells to achieve the same level of lysis. This corresponds to a five- to 10-fold increase in activity.

24 h preincubation. In a rat (Gross-virus-induced) tumor system, Ortiz de Landazuri and Herbermann (1972) have demonstrated an increase in cytotoxic activity of immune spleen cells after preincubation for 18-24 h.

In order to evaluate the effect of preincubation, 20×10^6 spleen cells collected 7 days after the second injection of irradiated EL4 cells were incubated at 37° C for 24 h in 20 ml DMEM 10%

TABLE I

CYTOTOXICITY OF IMMUNE SPLEEN CELLS: EFFECT OF BSA-GRADIENT SEPARATION AND/OR 24 h PREINCUBATION

Exp.	Treatment of immune cells:	Percentage specific ^{51}Cr release from EL4 target cells [1]											
		Untreated			BSA-grad. separated [2]			24 h 37° C pre-incub. [3]			24 h 37° C BSA-grad. separated [4]		
	Immune:target-cell ratios:	100	30	10	100	30	10	100	30	10	100	30	10
1		8	3	2	—	—	—	21	14	10	—	—	—
2		8	5	2	—	—	—	22	13	5	—	—	—
3		13	10	5	—	—	—	37	25	12	—	—	—
4		8	5	3	19	14	8	18	10	8	—	—	—
5		10	6	3	20	15	10	—	—	—	—	—	—
6		9	5	2	21	14	10	18	12	6	40	22	12
7		15	10	6	25	20	14	29	19	11	60	38	20
8		—	—	—	—	—	—	27	14	9	55	29	19
9		10	8	6	—	—	—	27	20	9	45	37	30
10		10	6	4	—	—	—	27	20	15	50	24	17
11		10	6	3	—	—	—	23	18	14	59	—	39
	Mean:	10	6	4	21	16	10	25	17	10	51	36	23

[1] Spleen cells from B6 mice collected 7 days after second injection of irradiated EL4 cells and incubated for 20 h with ^{51}Cr-labelled EL4 target cells at the indicated spleen-cell:target-cell ratios.

[2] BSA-gradient fractionation and selection of low-density cells.

[3] Immune cells preincubated for 24 h at 37° C.

[4] Preincubation followed by BSA-gradient separation.

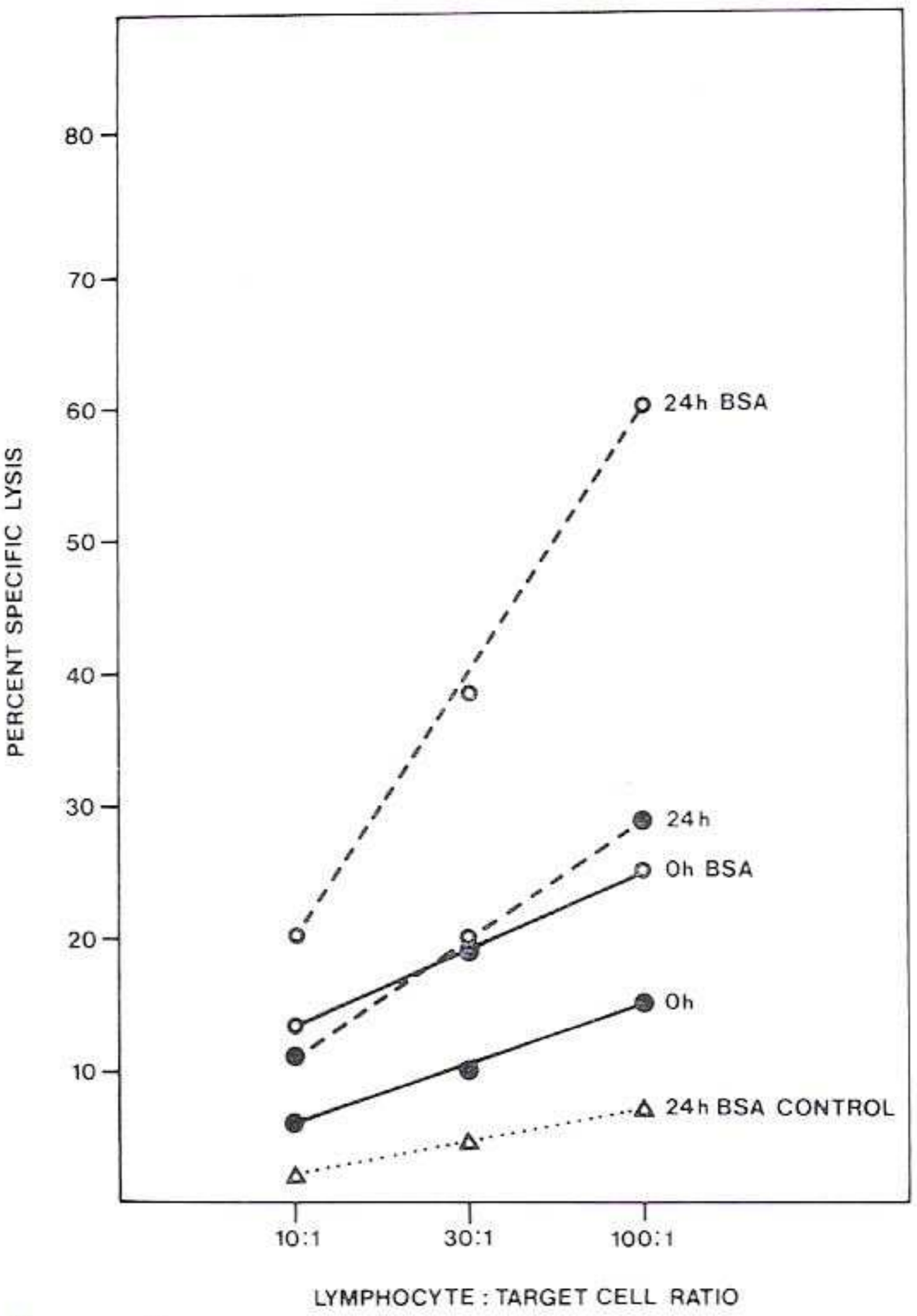

FIGURE 1

Comparison of the cytotoxic effect on ^{51}Cr labelled EL4 target cells of immune C57Bl/6 spleen cells obtained as indicated in Table I and tested (a) without pretreatment: ●——●, 0 h; (b) after BSA gradient separation: ○——○, 0 h BSA; (c) after 24 h preincubation at 37° C: ●– – –●, 24 h; (d) after 24 h preincubation followed by BSA gradient separation: ○– – –○ 24 h BSA. Cells of group (d) tested on unrelated P-815 target cells as a control (△· · · · ·△).

FBS. The recovery of lymphoid cells was approximately 70% of the original population. When tested for cytotoxicity, the preincubated cells regularly showed an increased lytic activity. In a representative experiment listed in Table I (exp. 7) the preincubated cells produced 15% specific target-cell lysis at a lymphocyte:target-cell ratio of 15:1, whereas the same population tested before incubation required a 100:1 ratio to produce the same amount of lysis. This corresponds to a six-fold increase in activity. In the seven comparative experiments shown in Table I, the average increase was approximately 5-fold.

As shown in two additional experiments (Table II), this increase in activity was temperature-dependent, *i.e.* immune spleen cells preincubated at 37° C showed increased activity, while the same cells maintained at 4° C exhibited a considerable loss of activity when compared to the original population assayed before incubation.

Combination of BSA-gradient separation and preincubation. Since both procedures, BSA-gradient purification and preincubation of immune spleen cells, resulted in increased cytotoxicity, it appeared possible that a successive application of both procedures, *i.e.* preincubation followed by BSA-gradient separation, would result in a cumulative increase in cytotoxic activity. As shown by the results of five such experiments presented in Table I, this was indeed the case. The results of experiment 7 (Table I), shown graphically in Figure 1, indicate that the spleen cells which had been preincubated for 24 h produced 29% specific lysis at a lymphocyte:target-cell ratio of 100:1, whereas the preincubated and BSA-gradient purified cells produced the same amount of lysis at a ratio of 15:1. Thus each procedure resulted in a six-fold enrichment, and the two-step procedure in a 36-fold increase.

24 h preincubation following removal of macrophages or high-density lymphocytes

In similar experiments, Ortiz de Landazuri and Herberman (1972) had shown that increased cytotoxicity following 18-24 h preincubation of immune spleen cells was dependent on the presence of adherent cells. The potential role of phagocytic cells in the generation of increased cytotoxicity by preincubation for 24 h was therefore investigated. Phagocytic cells were removed by treatment with carbonyl iron and these treated and untreated immune spleen cells were incubated for 24 h at 37° C. Cytotoxicity tests showed no significant difference in the increase in activity of the two populations (Table II), suggesting that phagocytic cells were not essential.

However, when immune spleen cells were fractionated on a BSA density gradient, and the low-density fraction collected and preincubated for 24 h at 37° C, the resulting cell population showed a greatly decreased lytic activity as compared to the unfractionated cells (Table II), demonstrating an important role of the high-density cells removed by the BSA gradient

TABLE II

INCREASED CYTOTOXICITY OF IMMUNE CELLS AFTER 24 h PREINCUBATION: TEMPERATURE DEPENDENCE AND EFFECT OF REMOVAL OF HIGH-DENSITY CELLS AND PHAGOCYTIC CELLS

Exp.	Source of immune cells	Pretreatment of immune cells	Percentage specific ^{51}Cr release from EL4 target cells in 20 h at immune:target-cell ratios of [1]		
			100	30	10
1	Spleen	None	15	10	6
		24 h 37° C	29	19	4
		24 h 4° C	17	8	4
2	Spleen	None	9	5	2
		24 h 37° C	18	12	6
		24 h 37° C—BSA-grad. [2]	40	22	12
		BSA-grad.—24 h 37° C [3]	8	4	2
3	Spleen	None	10	8	6
		24 h 37° C	27	20	15
		24 h 37° C—BSA-grad. [2]	45	37	30
		BSA-grad.—24 h 37° C [3]	13	7	4
4	Spleen	24 h 37° C	23	18	14
		Iron—24 h 37° C [5]	28	24	17
5	PC	24 h 37° C (non-adherent) [4]	47	43	33
		Iron—24 h 37° C [5]	44	36	31

[1] Immune cells obtained as indicated in Table I.
[2] Selection of low-density fraction after 24 h preincubation.
[3] Preincubation of low-density fraction after removal of high-density fraction.
[4] Preincubation followed by collection of non-adherent cells.
[5] Preincubation after removal of phagocytic cells.

fractionation in the generation of increased cytotoxicity. Parallel experiments confirmed that preincubation of unfractionated cells followed by BSA gradient separation resulted in a cumulative increase in cytotoxic activity (Table II).

Cytotoxicity of immune peritoneal cells

Earlier studies had shown that the peritoneal cells of mice immunized IP with syngeneic or allogeneic tumor cells showed higher cytotoxic activity than spleen cells (Brunner and Cerottini, 1971). Similarly, in the present system, the peritoneal cells from B6 mice immunized with two injections of irradiated syngeneic EL4 cells were found to be approximately 10 times more cytotoxic than spleen cells from the same animals (Fig. 1).

Removal of phagocytic cells. When phagocytic cells representing approximately 50% of the immune peritoneal cell population were removed by treatment with carbonyl iron, cytotoxicity was strongly increased. As shown in Table III and in Figure 2, a three- to five-fold increase in cytotoxicity over the non-purified population was observed. Since 40-50% of the cells were recovered after carbonyl iron treatment, this increase was higher than that expected after mere removal of irrelevant cells.

24 h incubation. A comparable increase in cytotoxicity was also observed when the adherent cells were removed by incubation of the immune peritoneal cells in flat-bottomed glass tubes for 24 h at 37° C. The unattached cells, when collected and tested against labelled target EL4 cells, showed an approximately three- to five-fold increase in specific lysis as compared to the total population tested before incubation (Fig. 2, Table III). Since removal of phagocytic cells before preincubation was almost as effective as removal of adherent cells after preincubation, the results suggested that it was the removal of phagocytic cells and not the preincubation which was the main cause of the increased lytic activity. This impression was confirmed by an experiment showing that removal of phagocytic cells by carbonyl iron treatment followed by 24 h pre-

incubation resulted in a population with similar cytotoxic activity to that obtained by preincubating the original population and subsequently removing adherent cells (Table II).

Effect of treatment with anti-theta serum

In attempts to demonstrate that the cytotoxicity of the preincubated and/or separated cell populations was mediated by T-cells, appropriate immune spleen and peritoneal cells were treated with anti-θ serum and complement. As shown in Table IV, anti-θ treatment caused a profound decrease in the cytotoxic activity of the tested effector cells. Control experiments included treatment of the immune cells with complement and normal AKR serum or incorporation of anti-θ serum into the assay medium. As shown in Table IV, both procedures had no effect on cytotoxicity.

TABLE III

CYTOTOXICITY OF IMMUNE PERITONEAL CELLS (PC): EFFECT OF REMOVAL OF PHAGOCYTIC CELLS OR PREINCUBATION

Exp.	Pretreatment of immune PC:	Percentage specific ^{51}Cr release from EL4 target cells in 20 h								
		Untreated			Carbonyl iron [1]			24 h 37° C (non-adherent cells) [2]		
	Immune:target-cell ratios:	100	30	10	100	30	10	100	30	10
1		19	9	4	—	—	—	53	40	15
2		24	17	12	—	—	—	—	—	—
3		42	26	18	56	42	24	—	—	—
4		20	10	8	50	20	16	—	—	—
5		30	24	17	45	36	28	50	39	28
6		—	—	—	—	—	—	53	48	12
7		—	—	—	—	—	—	58	43	34
8		21	15	8	—	—	—	47	43	33
	Mean:	26	17	11	50	33	23	52	43	27

[1] Removal of phagocytic cells.
[2] Preincubation followed by collection of non-adherent cells.

TABLE IV

EFFECT OF ANTI-θ SERUM TREATMENT ON THE CYTOTOXICITY OF PREINCUBATED OR BSA-GRADIENT SEPARATED IMMUNE CELLS

Immune cells, pretreatment [1]	Treatment of immune cells	Percentage specific ^{51}Cr release from EL4 target cells in 20 h at immune:target-cell ratios of		
		100	30	10
Spleen, 24 h 37° C	N. AKR+C	36	30	25
Spleen, 24 h 37° C	Anti-θ+C	0	0	0
Spleen, 24 h 37° C, then BSA-grad.	None	59	48	39
Spleen, 24 h 37° C, then BSA-grad.	N. AKR+C	60	51	42
Spleen, 24 h 37° C, then BSA-grad.	Anti-θ+C	1	0	0
Spleen, 24 h 37° C, then BSA-grad.	Anti-θ ser. added [2]	57	46	40
PC, 24 h 37° C (non-adherent cells)	None	52	22	11
PC, 24 h 37° C (non-adherent cells)	N. AKR+C	—	22	8
PC, 24 h 37° C (non-adherent cells)	Anti-θ+C	0	0	0

[1] Pretreatment as indicated in Tables I and II.
[2] Anti-θ serum (final dilution 1:10) added to the lymphocyte:target-cell mixture at the onset of the 20 h cytotoxic assay period.

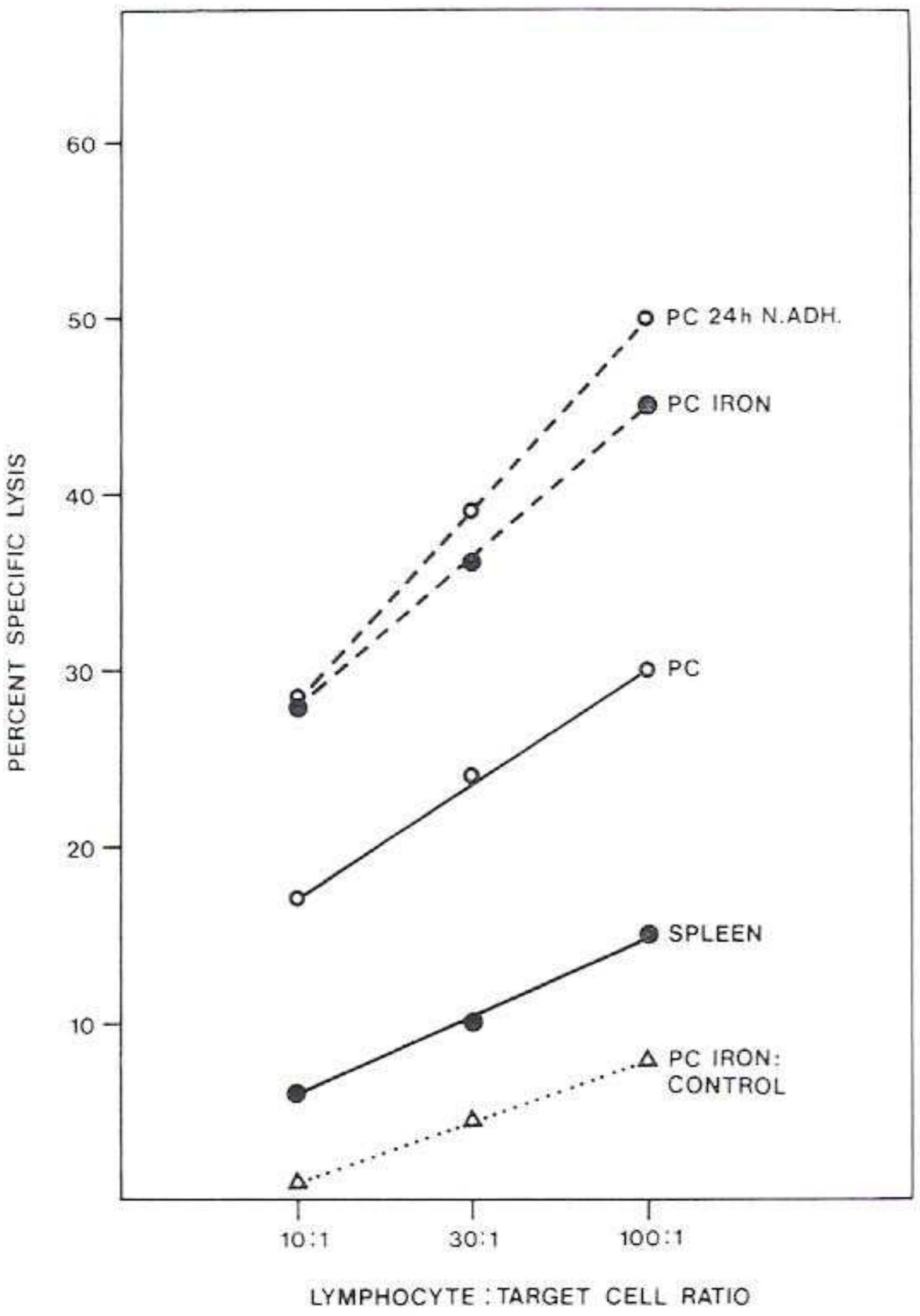

FIGURE 2

Comparison of the cytotoxic effect on ^{51}Cr-labelled EL4 target cells of immune peritoneal cells (PC) or spleen cells obtained as indicated in Table I and tested (a) without pretreatment: ●——● spleen; (b) without pretreatment: ○——○ PC; (c) after removal of phagocytic cells with carbonyl iron: ●– – –● PC iron; (d) after 24 h preincubation followed by removal of adherent cells: ○– – –○ PC 24 h non-adherent. Cells of group (c) tested on unrelated P-815 target cells as a control (△· · · · ·△).

Effect of Ig-anti-Ig column filtration

In order to exclude a participation of non-T cells in the cytotoxicity of preincubated and/or separated immune cell populations, Ig-bearing and/or Fc-receptor-bearing lymphocytes were removed by passage through an Ig-anti-Ig column (Wigzell and Andersson, 1971). Spleen cells from twice immunized animals were first preincubated for 24 h, then separated on a BSA gradient and finally passed through the Sephadex-Ig-anti-Ig column. The recovery was 30% of input and the cells in the eluate were found to contain less than 0.5% cells positive for surface immunoglobulins as tested by immunofluorescence. When this purified population was tested for cytotoxicity, a slight increase in activity was observed (Table V). As a control, similarly pretreated immune spleen cells were passed through a column containing Sephadex-Ig treated with normal rabbit serum. Here the recovery was 75%, and 33% of the eluted cells carried Ig markers as detected by immunofluorescence. These cells, however, had the same cytotoxic activity as did those of the original population (Table V).

TABLE V

EFFECT OF IG-ANTI-IG COLUMN FILTRATION ON THE CYTOTOXICITY OF PREINCUBATED AND BSA-GRADIENT-SEPARATED IMMUNE SPLEEN CELLS

Column filtration [1] of immune cells	Ig. pos. cells [2]	Percentage specific ^{51}Cr release from EL4 target cells in 20 h at immune: target-cell ratios of		
		100	30	10
None	36%	59	48	39
Ig-anti-Ig	0.5%	69	65	59
Ig NRS	33%	62	58	51

[1] The immune spleen cells were preincubated for 24 h at 37° C followed by BSA-gradient separation and collection of low-density cells, and then passed through an immunoadsorbent column of Sephadex mouse Ig-rabbit anti-mouse Ig. The control column was prepared with mouse Ig-normal rabbit serum.

[2] Determined by direct immunofluorescent test.

In vivo *protection tests*

In order to answer the question of the functional *in vivo* activity of the preincubated and BSA-gradient-separated immune cells, *in vivo* protection tests were carried out. The pretreated spleen cells, collected and processed 7 days after secondary immunization, were mixed with EL4 tumor cells at ratios of 1,000:1 and 100:1, and injected subcutaneously into B6 recipients. As shown in Table VI, the purified lymphoid cells were more effective in protecting the syngeneic animals from EL4 tumor challenge than the original population. Immune spleen cells preincubated for 24 h and then selected on a BSA gradient gave complete protection at an effector-to target-cell ratio of 1,000:1 while protecting four out of five animals at a ratio of 100:1. At 1,000:1, preincubated immune cells also protected four out of five animals, whereas the original immune population protected three out of five test animals.

TABLE VI

PROTECTIVE EFFECT OF PRETREATED IMMUNE CELLS *IN VITRO*

Number of cells mixed with 10^3 EL4 cells and injected SC [1]	Source of cells injected [2]	Result day 15		Result day 30	
		Survivors/ total	Mean tumor ∅ in mm	Survivors/ total	Mean tumor ∅ in mm
10^6	Normal spleen	5/5	21	0/5	—
10^6	Immune spleen	5/5	6	2/5	23
10^6	Immune spleen, 24 h 37° C	5/5	1	4/5	11
10^5	Immune spleen, 24 h 37° C-BSA-grad.	5/5	1	4/5	5
10^6	Immune spleen, 24 h 37° C-BSA-grad.	5/5	0	5/5	0
10^6	Immune PC iron treated	5/5	0	4/5	5
10^7	Immune PC iron treated	5/5	0	5/5	0

[1] Normal or immune spleen cells or PC were mixed with 10^3 viable EL4 cells and injected SC into normal B6 mice. 10^2 EL4 cells killed all injected mice within 30 days (data not shown).
[2] Pretreatment as indicated in Tables I and II.

Specificity tests

In order to rule out the possibility that the cytotoxic activity of the purified and/or preincubated lymphoid cell populations was non-specific, their lytic effect was tested in a number of experiments against the unrelated P-815 (DBA/2) mastocytoma cells known to be highly sensitive to lysis by specifically sensitized lymphocytes. As shown in Table VII and Figures 1 and 2, only minimal cytotoxic activity against these allogeneic cells could be demonstrated.

DISCUSSION

The present work demonstrates that the lytic activity, as tested *in vitro*, of immune spleen and peritoneal cells of inbred B6 mice immunized with the syngeneic EL4 lymphoma can be considerably increased by appropriate fractionation procedures. They also confirm the observations made by Ortiz de Landazuri and Herberman (1972) showing that preincubation of immune lymphoid cells at 37° C for 24 h may result in increased cytotoxicity.

TABLE VII

SPECIFICITY OF CYTOTOXIC EFFECT OF PRETREATED IMMUNE CELLS TESTED AGAINST EL4 AND P-815 (ALLOGENEIC) TARGET CELLS

Exp.	Source of immune cells	Pretreatment of immune cells [1]	Percentage specific ^{51}Cr release					
			From EL4 target cells at immune:target-cell ratios			From P-815 target cells at immune:target-cell ratios		
			100	30	10	100	30	10
1	Spleen	None	9	5	2	8	3	0
	Spleen	24 h 37° C	21	14	10	6	1	0
2	Spleen	24 h 37° C	32	18	12	4	0	0
3	Spleen	24 h 37° C	27	20	15	8	6	5
	Spleen	24 h 37° C-BSA-grad.	55	29	19	7	4	2
4	Spleen	24 h 37° C-BSA-grad.	45	37	30	12	11	9
5	PC	Iron	—	31	10	—	3	0
6	PC	Iron	—	81	56	—	28	10
	PC	24 h 37° C (non-adherent cells)	—	42	24	—	6	1
7	PC	24 h 37° C (non-adherent cells)	50	14	8	8	1	0
8	PC	24 h 37° C (non-adherent cells)	53	48	12	15	8	4

[1] Pretreatment as indicated in Tables I and II.
[2] Immune cells obtained as indicated in Table I.

Increased cytotoxicity of low-density cell populations selected for by density-gradient separation confirmed earlier experiments in allogeneic systems which showed that in the early phases of the immune response to allogeneic tumor cells, low-density lymphocytes contained higher relative numbers of cytotoxic lymphocytes than high-density cells (Pelet *et al.*, 1971; Shortman *et al.*, 1972). The low-density cells are presumably lymphoblasts generated in response to specific antigenic stimulation.

As shown in Table I and Figure 1, the lymphocyte fraction containing cells with a density of 1.08 g/cm^3 or less was five to 10 times more active than the initial population. A similar increase was noted when immune spleen cells which had been preincubated for 24 h at 37° C were subsequently purified by BSA density-gradient fractionation. Hence, it could be shown that a two-step procedure involving preincubation followed by BSA-gradient purification led to a cumulative 30- to 100-fold increase in cytotoxicity.

Several explanations for the increased cytotoxicity observed after preincubation of immune spleen cells for 24 h may be put forward. They include (1) preferential death or loss of irrelevant or inhibitory cells with the concomitant persistence of active cells, (2) generation of CTL from precursor cells, a mechanism which may depend on the presence of macrophages, and (3) the removal of blocking factors, including antigen, antibody or immune complexes. Death of irrelevant cells alone does not explain the five-fold increase in cytotoxicity observed, since only about 30% of the cells were dead by the end of the 24 h preincubation period. Preferential death or loss of inhibitory cells cannot be formally excluded as a contributing factor. Antibody-forming cells could be inhibitory by generating blocking antibody or complexes. Similarly, our own results showing that macrophages present in immune peritoneal cells may inhibit cytotoxicity suggest that a loss of inhibitory adherent cells during preincubation could account for such an increase. However, this appeared unlikely since the removal of the relatively low number of phagocytic cells present in immune spleen-cell populations did not result in a significant direct increase in cytotoxicity (unpublished results).

On the other hand, high-density lymphocytes were found to be necessary for the observed augmentation of cytotoxic activity. Low-density spleen cells recovered from a BSA density gradient and then preincubated for 24 h showed a considerably decreased activity as compared to unfractionated cells (Table II). This is compatible with, but does not prove, generation of CTL from high-density precursor cells. That the generation of CTL from precursor cells within 24 h is possible is shown by recent studies on the mechanism of CTL induction in mixed lymphocyte cultures (MacDonald *et al.*, 1974). Generation of low-density CTL is also suggested by the results showing that the low-density fractions obtained by BSA-gradient purification after preincubation contained relatively more CTL than the low-density fractions obtained before incubation (Table I).

The necessity for the presence of adherent cells as reported by Ortiz de Landazuri and Herberman (1972) was not confirmed in the present system. Spleen cells treated with carbonyl iron and subsequently incubated for 24 h showed essentially the same increased activity as the cells preincubated without such a treatment. This suggests that, if the presence of phagocytic cells was required for increased cytotoxicity, the carbonyl iron treatment was ineffective in removing the appropriate cell population. Finally, blocking of the specific receptors on CTL by antigen or complexes and release of these blocking factors during preincubation must be considered as a possible explanation. Currie and Basham (1972) have reported that the extensive washing of lymphocytes from cancer patients greatly enhanced their specific cytotoxic effect. In the present system, however, extensive washing did not increase their cytotoxicity (unpublished results). These results could be explained by a lack of involvement of inhibitory factors in our system, or, if present, a firmer attachment to the cell surface. Experiments to test the possibility that such factors are released into the supernatant fluid during the 24 h preincubation period are now in progress.

Peritoneal cells from mice immunized by the intraperitoneal injection of syngeneic or allogeneic tumor cells have been shown by several investigators to have a higher cytotoxic activity *in vitro* than spleen cells from the same animals (Brunner and Cerottini, 1971; Berke *et al.*, 1972). In the present system, these observations were confirmed. In fact, removal of phagocytic cells with carbonyl iron resulted in an increase in

cytotoxicity which surpassed that expected by the mere removal of irrelevant cells. Since approximately 50% of the immune peritoneal population were phagocytic cells, and 40-50% of the original cell population was recovered after carbonyl iron treatment, a two-fold increase in cytotoxicity could be expected. The observed three- to five-fold increase may therefore be due to the removal of inhibitory phagocytic cells.

The original peritoneal cell population from which the phagocytic cells had been removed with carbonyl iron showed almost the same increased cytotoxicity as the non-adherent cells recovered after 24 hours' preincubation. Similarly, preincubation after removal of phagocytic cells yielded populations with similar activity to that obtained by removal of adherent cells after preincubation. This suggests that it was not the preincubation but the removal of adherent or phagocytic cells which was mainly responsible for the increased activity. Such a result would agree with the concept that immune spleen cells contain sensitized precursor cells which can differentiate during preincubation to form CTL, while most of the sensitized lymphocytes present in immune peritoneal cell populations are already fully differentiated.

In attempts to identify the cells which were active in the purified and/or preincubated cell populations, the processed cells were treated with anti-θ serum and complement, or were passed through Ig-anti-Ig columns to remove cells carrying surface Ig and/or Fc receptors. After treatment with anti-θ serum and complement the cytotoxic activity was totally abolished. On the contrary, no reduction in the activity was observed after removal of cells carrying Ig or Fc receptors. Removal of phagocytic or adherent cells from the peritoneal cell populations actually increased their activity while the level of cytotoxicity of the spleen cells was the same before and after the treatment with carbonyl iron. These results, taken together, suggest that the cytotoxic reaction described in these experiments was predominantly T-cell-dependent. Our observations are thus in accordance with those obtained for the syngeneic murine sarcoma virus (MSV)-induced tumor system (Plata *et al.*, 1974).

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DÉTECTION DE LYMPHOCYTES T CYTOTOXIQUES DANS LE SYSTÈME DE LA LEUCÉMIE MURINE EL4: AUGMENTATION DE L'ACTIVITÉ DES CELLULES SPLÉNIQUES ET PÉRITONÉALES IMMUNES A LA SUITE D'UNE PRÉINCUBATION ET DE DIFFÉRENTS PROCÉDÉS DE FRACTIONNEMENT CELLULAIRE

L'activité cytotoxique des cellules spléniques et péritonéales de souris C57Bl/6 injectées avec des cellules leucémiques syngéniques irradiées EL4 a été testée in vitro *à l'aide de cellules cibles marquées au* ^{51}Cr. *Cette activité a été mesurée avant et après l'utilisation de diverses méthodes de séparation destinées à enrichir les populations cellulaires testées en cellules effectrices. Au pic de la réponse, les cellules spléniques immunes avaient une activité cytotoxique très faible. Le fractionnement de cette population sur un gradient de densité a permis de la séparer en deux fractions, dont l'une, contenant des cellules lymphoïdes de faible densité (inférieure à 1.08 g/cm³) a montré une activité cytotoxique 5 à 10 fois plus grande que la population originale. Une augmentation de l'activité cytotoxique a également été observée à la suite de la préincubation des cellules spléniques immunes pendant 24 heures à 37° C, confirmant les résultats de Ortiz de Landazuri et Herberman (1972). L'utilisation successive d'une préincubation suivie d'une séparation sur gradient a eu comme résultat une augmentation*

cumulative de 30 à 100 fois de la cytotoxicité. L'élimination des cellules de forte densité avant la préincubation a entraîné une forte diminution de l'activité cytotoxique, suggérant la formation de lymphocytes cytotoxiques à partir de précurseurs plus denses pendant la période de préincubation. Les cellules péritonéales immunes ont montré une activité cytotoxique 3 à 5 fois plus élevée à la suite (1) d'une élimination des cellules phagocytaires, (2) d'une préincubation pendant 24 heures suivie d'une élimination des cellules adhérentes, et (3) de l'élimination des cellules phagocytaires suivie d'une préincubation pendant 24 heures. Ces résultats suggèrent que les cellules adhérentes n'étaient pas cytotoxiques mais semblaient plutôt inhiber l'activité cytotoxique des cellules péritonéales immunes. Les résultats obtenus après traitement des populations immunes avec un antisérum anti-0 et du complément, et après leur passage sur une colonne d'immunoglobulines-anti-immunoglobulines, a démontré que les cellules effectrices cytotoxiques étaient des cellules T. Enfin, des études in vivo *ont montré une activité protectrice accrue des cellules spléniques immunes prétraitées par rapport à la population originale.*

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