Detection of cytotoxic T-Lymphocytes in tumour bearing animals: procedures to isolate highly active populations

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The spleen cells from lymphoma bearing DBA mice were tested for their cytotoxic activity against the labelled target cells. The very low levels of the activity of the Cytotoxic T Lymphocytes in the spleens from the tumour bearing mice could be functionally enriched by a combination of the following three steps : (a) pre-incubation of spleen cells for 24 hr. at 37°C, (b) density gradient separation and selection of low density lymphocytes, (density less than 1.08 gm/cm³) and (c) velocity sedimentation technique, thereby selecting the larger sized lymphocytes (cell diameter 10 μ). Even though this highly active population of cells constituted only 0.25% of the original spleen cells, they showed about thousand times increase in cytotoxic activity. The effector cells are predominantly T cells. Pre-incubation procedure may remove blocking factors from the surface of the lymphocytes. The isolation of highly active tumourocidal lymphocytes by a step-by-step enrichment procedure may have important applications in future immunotherapeutic approaches to cancer.

INTRODUCTION

CELL mediated cytolysis of tumour cells in the host may depend on many mechanisms such as (a) Killing by cytotoxic T lymphocytes (CTL), (b) antibody dependent killing by normal non-T cells (K cells) and (c) killing by armed macrophages (reviewed by Certottini and Brunner, 1974). However, in weak syngeneic tumour systems it is often difficult to detect cytotoxicity of the immune lymphocytes *in vitro* by the present available methods. This may be due to the relative low frequency of the effector cells or the presence of interfering mechanisms. Recent studies have shown that CTL could be enriched by simple physical methods in allogeneic systems (Shortman *et al* 1972). In the early phase of the immune response CTL are predominantly large cells (Mac Donald *et al*, 1973) and are of low-density (Pelet *et al*, 1971). Based on these principles, Vasudevan *et al*, 1974) have shown that CTL from spleen or peritoneal cavity of C 57 BL mice immunised with a syngeneic non-viable tumour (EL4) cells could be enriched up to 100 times. The aim of the present study is to extend these principles into tumour bearing animals. It will be shown that even though CTL from tumour-bearing mice were very weakly cytotoxic, the activity could be enriched by successive stages of physical purification procedures.

Animals : Five to six months old DBA male mice were used for the present study. Each test group consisted of 5 animals.

Tumour : Lymphoma, spontaneously generated in the DBA colony has been maintained by intraperitoneal or subcutaneous passage.

Tumour Induction : 1 x 10^3 viable lymphoma cells were injected subcutaneously into DBA mice. All animals developed palpable tumour at the injection site by 7-10 days, and died around 22-28 days.

Carbonyl Iron Treatment : To 100 x 10^6 spleen cells in 20 ml DMEM with 10% foetal calf serum (FCS), 250 mg of carbonyl iron (particle size 3.5μ) were added and incubated for 30 min. at 37°C. The iron particles were then removed by the addition of a magnetic stirring bar. This procedure will remove more than 99% of the macrophage-like cells.

Density Gradient Separation : The method has been described originally by Shortman *et al*, (1972) and modified by Vasudevan *et al*, (1974). Briefly 100 x 10^6 spleen cells in 0.1 ml DMEM were dispersed at 4°C in 1 ml of 27% Bovine Serum Albumin (BSA) solution, having a density of 1.08 gm/cm^3, pH 7.1, and 308 m. osmil. Then 1 ml. of 30% BSA solution was layered underneath and 1 ml. of 10% of BSA solution was layered on the top of the cell suspension. The tube was centrifuged at 3000 x g for 10 min at 4°C. The cells in the middle layer of the gradient (having density less than 1.08 gm/cm^3) were taken and washed twice in DMEM. These low-density lymphocytes will be roughly 18% of the original lymphocyte count.

SEPARATION OF CELLS BY VELOCITY SEDIMENTATION

The method has been modified from Miller and Phillips (1969). Briefly, spleen cells were layered as a thin band, on the top of a shallow continuous gradient of 3% to 30% FCS in DMEM at 4°C, and allowed to sediment at 1 x g for 4 hr. The function of the gradient is to prevent convection and consequent mixing. Separation of cells in different bands takes place mainly on the basis of the size of the cells. The following equation has been found to hold good in these experimental conditions :

$$S \simeq \frac{r^2}{4}$$

where S is the sedimentation velocity in mm/hr and r is the radius of the cell. Thus the larger sized cells will have a greater sedimentation rate.

Immuno Adsorption Column : Columns were prepared as described by Schlossman and Hudson (1973) with minor modifications (Vasudevan *et al*, 1974). In principle, activated Sephadex was mixed with normal mouse immunoglobulin; excess Ig was washed out; then rabbit anti-mouse-Ig was added, and washed. When the spleen cells were passed through this column, the Ig-bearing cells (B cells) and cells bearing Fc markers (K cells) should have adhered to the column, and so the eluted cells will be enriched in T cells.

Cytotoxicity Test (Brunner Assay) : The method has been described in detail by Cerottini and Brunner (1971), which has been modified to suit the syngeneic systems (Vasudevan *et al*, 1974). To summarize, ^{51}Cr labelled target cells were incubated with spleen cells for 20-24 hr. at 37°C. The release of ^{51}Cr will be proportional to the lysis of the target cells. The percentage of specific ^{51}Cr release was calculated as :

$$\frac{\left(\begin{array}{l}\text{}^{51}\text{Cr release in the}\\ \text{presence of}\\ \text{immune lymphoid}\\ \text{cells}\end{array}\right) - \left(\begin{array}{l}\text{}^{51}\text{Cr release in the}\\ \text{presence of normal}\\ \text{lymphoid cells}\end{array}\right)}{\left(\begin{array}{l}\text{Maximum}\\ \text{releasable } ^{51}\text{Cr}\end{array}\right) - \left(\begin{array}{l}\text{}^{51}\text{Cr release in the}\\ \text{presence of normal}\\ \text{lymphoid cells}\end{array}\right)} \times 100$$

RESULTS

Immune Status in Tumour Bearing Animals

A group of DBA mice were injected with syngeneic lymphoma cells, and on different days, some of them were sacrificed, and spleen cells were tested for their cytotoxic activity against the target lymphoma cells. A typical experiment is shown in graphical form in Fig. 1. It was seen that when the spleen cells were directly tested, no significant cytotoxic activity was detected in any of the days tested. However, when the spleen cells were pre-incubated at 37°C for 24 hr. and selected by BSA gradient and then tested against the target lymphoma cells, the cytotoxicity was detected on 7th day of the intake of tumour, reaching a peak on the 15th day, and then declining to very low levels by the 22nd day. It was also observed that the decreased cytotoxicity coincided with the increased growth of the tumour.

Methods to Increase the Cytolytic Effect of the Immune Spleen Cells

(a) **24 hr. Pre-Incubation :** 20 x 10^6 spleen cells from the tumour-bearing animals were pre-incubated at 37°C in 5% CO_2 atmosphere for 24 hr. in 20 ml DMEM with 10% FCS. The recovery of lymphoid cells was approximately 75%. This procedure was very effective in increasing the cytotoxic activity of the spleen cells. The results of a typical experiment is shown in Figure 2.

FIGURE.1

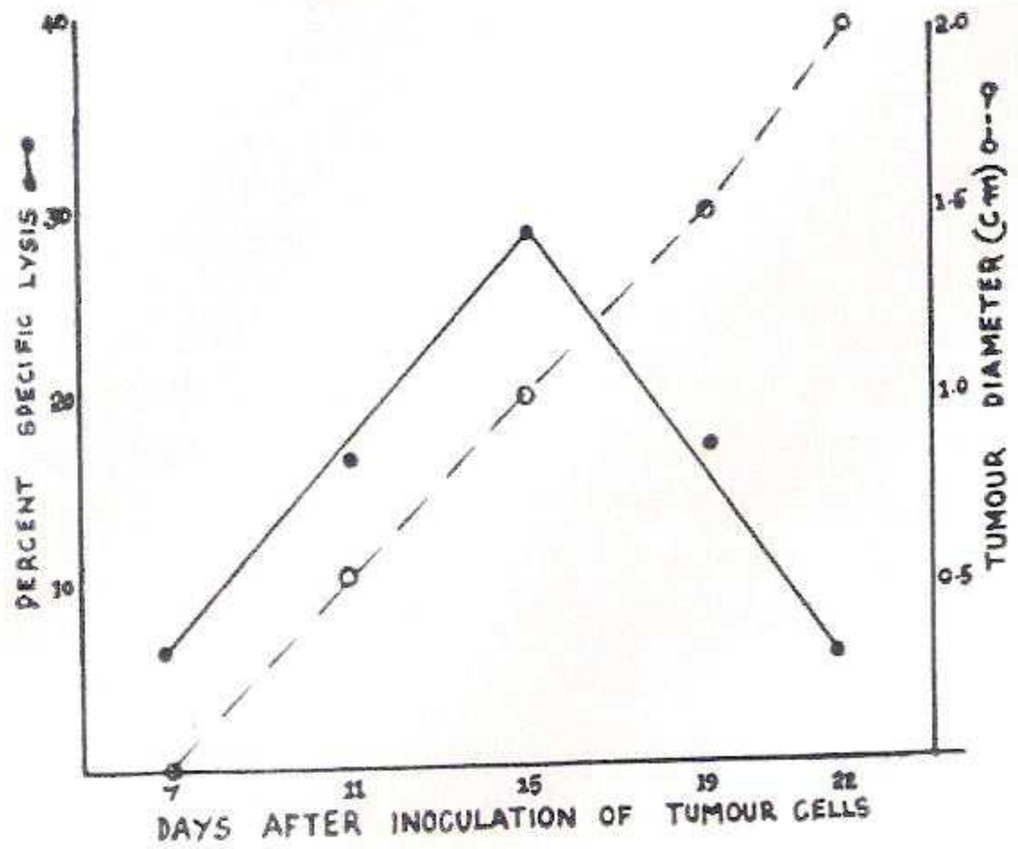

Fig. 1. DBA mice were given subcutaneous injection of 10^3 viable syngeneic lymphoma cells. The spleen cells were harvested at different time intervals after the tumour inoculation. These spleen cells were pre-incubated for 24 hr at 37°C, followed by BSA—gradient separation of low density (less that 1.08 g/cm³) cells. These highly active spleen cells were assayed for their cytotoxic effect against Cr-labelled lymphoma target cells, at a spleen cell to target cell ration of 100:1.

●—● *Percentage of specific lysis.*

O....O *Tumour growth.*

FIGURE.2

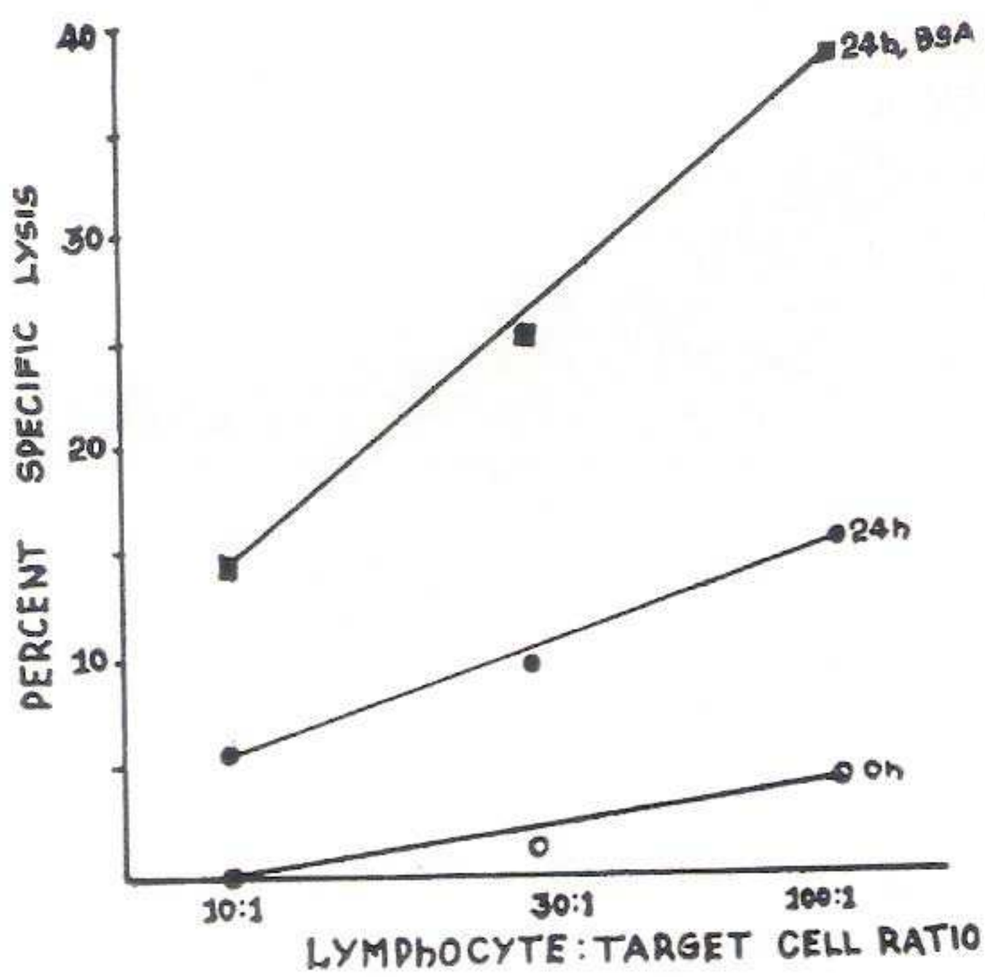

Fig. 2. Comparison of the cytotoxic effect on ^{51}Cr labelled lymphoma target cells by immune spleen cells from tumour bearing DBA mice. Results are the mean values obtained from the ten tests shown in Table 1.

0 hr = Spleen cells without any treatment.

24 hr = Spleen cells were pre-incubated for 24 hr at 37°C before doing the assay.

24 hr, BSA = Spleen cells pre-incubated for 24 hr, followed by BSA—gradient separation, and then the assay was done. BSA—gradient technique separates the low-density lymphocytes, the method is described in detail in the text.

(b) **BSA Gradient Separation :** When the pre-incubated spleen cells were further enriched by the BSA-gradient centrifugation, such that cells having a density less than 1.08 g/cm³ were selected, the cytotoxicity was further increased to ten-times or more (Fig. 2).

(c) **Velocity Sedimentation Technique :** Spleen cells from tumour-bearing animals on day 15 were pre-incubated for 24 hrs. at 37°C; further, low-density lymphocytes were separated by BSA gradient method and then those cells were finally fractionated by the velocity sedimentation technique. The different fractions, obtained from the last procedure were then tested for their cytolytic activity against the target lymphoma cells. The results of a typical experiment is shown in Figure 3. It was observed

FIGURE.3

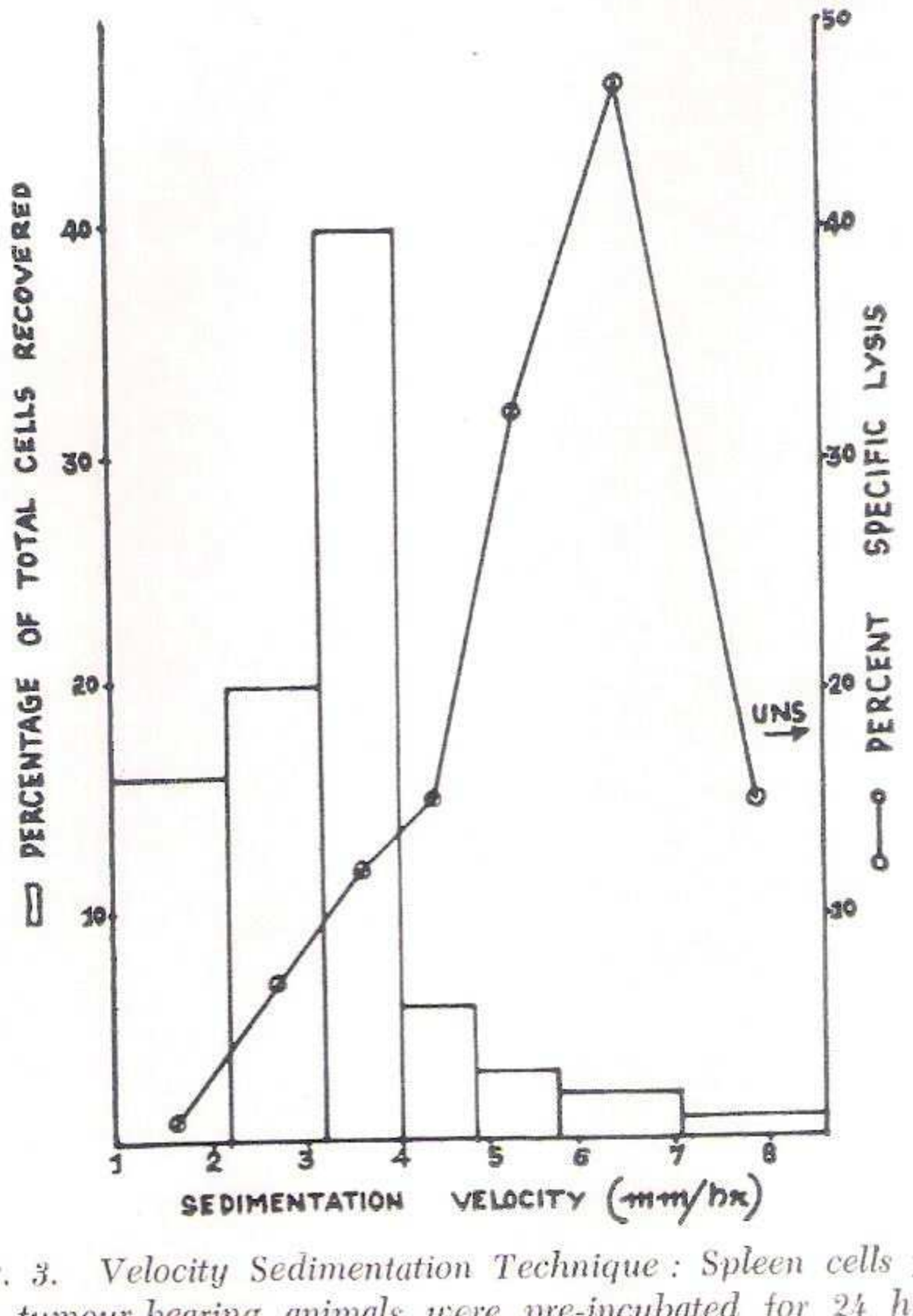

Fig. 3. Velocity Sedimentation Technique : Spleen cells from the tumour-bearing animals were pre-incubated for 24 hr. at 37°C, and then the low-density lymphocytes were selected by the BSA gradient method. These purified population was then separated into different fractions depending on their sedimentation velocity (which in turn depends mainly on the cell size). These different fractions are represented as the bars. Each of these fractions were tested for their cytotoxic activity against the labelled target lymphoma cells. The assay mixture contained lymphocytes; target cells at a ratio of 10:1. The specific cytolytic activity of each fraction is represented in the graph (0—0).

UNS = The level of cytolytic activity of the unseparated population.

that the fraction having a mean sedimentation velocity of 6.3 mm/hr. (having a mean cell diameter of 10 μ) had the highest cytotoxic activity. This cell population constituted only 0.25% of the original spleen cell population.

A comparison of these three steps of enrichment procedures are shown in Fig. 4. The most active population after the successive three-step procedure could cause 20% chromium release even at an effector: target ratio of 1 : 1. To get the same percentage of 51_{cr} release, the pre-incubated and BSA gradient separated cells needed an effector : target cell ratio of 10 : 1, while the pre-incubated lymphocytes required 100 : 1 ratio to get the same activity. It is interesting to note that the original untreated spleen cells produced ony 2% chromium release at a lymphocyte : target cell ratio of 100 : 1, whereas the highly active population (pre-incubation + BSA + velocity sedimentation) caused 20% release even at a 1 : 1 ratio. Thus, there is approximately a thousand-fold enrichment in the cytotoxic activity after the three-step isolation procedure.

In order to understand the mechanism of the activation of lymphocytes during the pre-incubation procedure, a series of experiments were conducted : It is observed that this effect is temperature dependent. The activation took place when incubated at 37°C, but not at 4°C (Table I Expt. No. 1). However, the activation needed the participation of cells having high density. Thus, low density lymphocytes fractionated from the pre-incubated cells, showed an increase in activity; but when the high density lymphocytes were excluded first, and then incubated for 24 hr. at 37°C, there was no increase in the activity (Table I, Expt. No. 2). Further, when the macrophages were removed by the carbonyl iron treatment and then the cells were pre-incubated, the cytotoxicity of the cells was unaltered (Table I, expt. No. 3). This shows that the activation process is not dependent on the presence of phagocytic cells. Moreover, the macrophages did not participate in the effector mechanism, as evidenced by the fact that removal of phagocytic cells did not decrease the chromium release from the target cells (Table I, Expt. No. 4).

Pre-incubation procedure was thus shown to increase the activity of the CTL. When the supernatant from this pre-incubation mixture was added to the chromium release assay medium, the activity was suppressed. Moreover, as the supernatant fluid was more and more concentrated, there was a corresponding increase in the abrogation of the cytotoxicity. (Table II). This shows that the spleen cells from the tumour bearing animals are suppressed by some factor, and this factor is shed into the culture medium during the 24 hr. pre-incubation procedure.

A few experiments were designed to assess what type of cells are participating in the cytolysis of target cells. The highly active effector cell population was passed through an immuno-adsorption column containing anti-mouse-Ig, thereby effectively removing the B cells and possibly the K cells from the population. This B-depleted effector cells had the same activity as that of the original population or of those cells passed through the control column containing no anti-Ig. (Table III). This shows that the CTL are predominantly cells having no Ig markers on their cell surface.

TABLE I

Augmentation of cytotoxicity by the Pre-incubation procedure

No. of experiment	Pretreatment of spleen cells	% of specific 51 Cr. release from the target lymphoma cells at the lymphocyte : target cell ratio of		
		100 : 1	30 : 1	10 : 1
1.	Nil	7	1	0
	24 hr incubation at 37°C	28	20	16
	24 hr incubation at 4°C	5	0	0
2.	Nil	6	1	0
	24 hr. at 37°C	10	6	4
	24 hr 37°C + BSA gradient (*)	54	35	21
	BSA gradient + 24 hr 37°C (**)	8	2	0
3.	Nil	5	0	0
	24 hr 37°C + BSA gradient (*)	42	27	15
	Iron + 24 hr 37°C + BSA gradient (***)	40	28	16
4.	Nil	6	3	0
	24 hr 37°C + BSA gradient	43	22	14
	24 hr 37°C + BSA gradient + Iron	45	24	16

(*) Spleen cells were pre-incubated for 24 hr at 37°C, and then the low density fraction was separated by Bovine Serum Albumin gradient.

(**) Low density fraction was first collected by the BSA gradient method, and then pre-incubated for 24 hr at 37°C, before the assessment of the cytotoxic activity.

(***) Phagocytic cells were removed by carbonyl — iron treatment then pre-incubated for 24 hr at 37°C, and then low density fraction was separated by BSA gradient, before the chromium release assay.

TABLE II

Effect of Supernatant fluid from the 24 hr pre-incubation mixture (*)

experiment No.	51 Cr. release assay medium containing	% of specific release of 51-Cr. at the effector : target cell ratio of		
		100 : 1	30 : 1	10 : 1
3	No supernatant	42	27	15
	Supernatant as such	45	26	17
	Supernatant X 5 times concentrated	30	22	15
	Superntant X 10 times Conc.	18	15	10
	Supernatant X 15 times conc.	13	10	8
	Supernatant X 20 times conc.	11	10	9
4	No supernatant	43	22	14
	Supernatant as such	40	21	14
	Supernatant X 10 times conc.	15	8	3
	Supernatant X 20 times conc.	6	2	0

(*) Spleen cells from tumour-bearing animals were pre-incubated for 24 hr. at 37°C. The Supernatant fluid from this pre-incubation mixture was concentrated by ultrafiltration (Amicon membrane) to the desired level. This supernatant was added to the incubation mixture containing chromium labelled target cells and the immune lymphocytes, pre-incubated and selected by BSA gradient.

TABLE III

Effect of filtration of immune cells through immuni-adsorbant column

	Purified effector cells passed through the immuno-adsorbent column containing	Percentage of specific release of chromium from the target lymphoma cells at the effector : target cell ratio of		
		100 : 1	30 : 1	10 : 1
(*)	None	61	54	48
	Rabbit-anti-mouse-Ig	64	53	45
(**)	Normal rabbit-Ig	63	54	47

(*) Spleen cells from tumour-bearing animals were pre-incubated for 24 hr. at 37°C, followed by BSA gradient separation, thus selecting the low-density cells. These highly active cells were tested for their cytotoxic activity.

(**) As a control, a column was prepared which contained Sephadex-mouse Ig-normal rabbit Ig. The highly active effector cells were passed through this column. This control column will not retain B cells. The eluted cells were tested for their cytotoxic activity.

DISCUSSION

A three-step combination procedure of pre-incubation for 24 hr., then BSA gradient separation and further isolation by velocity sedimentation resulted in a very dramatic increase in the cytotoxic activity of the spleen cells, if calculated in a cell basis (Fig. 4). It has previously been shown in allogeneic systems that CTL are predominantly larger cells (MacDonald *et al*, 1973).

In order to establish which type of effector cells were participating predominantly in the present assay system, a few experiments were carried out. Thus,

FIGURE.4

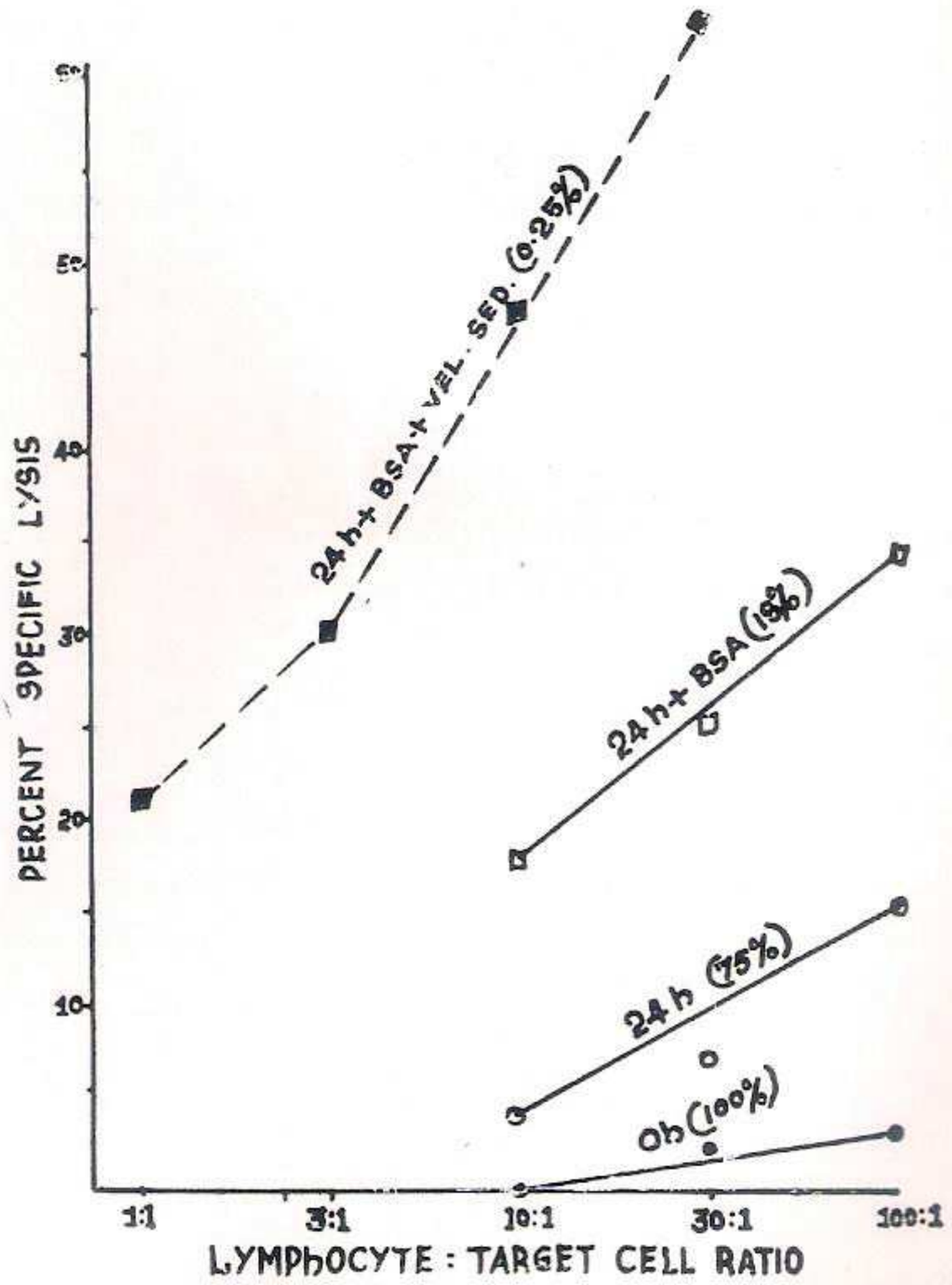

Fig. 4. Comparison of the effect of various fractionation procedures.

Spleen cells from the tumour-bearing animals were fractionated by different procedures, which are then mixed with chromium-labelled target lymphoma cells, and the percentage of specific release of chromium was assayed.

0 hr (●—●) = the values obtained when target cells were incubated with the immune spleen cells without any purification procedures.

24 hr (O———O) = Spleen cells were pre-incubated for 24 hr at 37°C, and then tested for their cytotoxic action against the target lymphoma cells. The recovery after the pre-incubation procedure is about 75% of the original spleen cell population.

24 hr + BSA (□—□) = Spleen cells were pre-incubated for 24 hr at 37°C and then the low density lymphocytes were selected by BSA-gradient method. This fraction constituted about 18% of the original spleen population. The above three lines in the graph represent the same values given as experiment No. 6 in Table No. I.

24hr + BSA + Vel. Sed. (■.....■) = Spleen cells were successively purified by pre-incubation for 24 hr at 37°C; then BSA-gradient separation and finally by velocity sedimentation technique. These highly cytotoxic fraction constituted only 0.25% of the total spleen cells.

removal of Ig-bearing cells or of cells carrying Fc-receptors by means of immunoadsorption column (Table III), or removal of phagocytic cells by the carbonyl iron treatment (Table I) did not reduce the cytolytic activity of the effector cells. These observations lead us to exclude the B cells, K cells and macrophages as possible candidates for the *'in vitro'* cytotoxic effect observed in the present study. By using anti-theta serum, it is now well established that the chromium release-assay method (Brunner system) generally indicates T cell activity (Cerottini and Brunner, 1974). These results, taken together, suggest that the cytotoxic reaction described in these experiments, was predominantly T cell dependent.

In the present study, the spleen cells from the tumour-bearing animals, kept at 37°C for 24 hr. were found to acquire increased cytolytic activity against target cells (Fig. 2). This is in line with the previous observations on animals immunized with syngeneic non-viable tumour cells (Vasudevan *et al*, 1974) or with virus induced tumour cells (Landazuri & Herberman, 1972). This phenomenon may be due to a) preferential death of irrelevant cells during the incubation. But this explanation alone is inadequate. Only about 25% of cells were dead during this incubation time, whereas the activity was increased atleast ten fold (Fig. 4).

b) Removal of inhibitory cells during the pre-incubation procedure.

c) Generation of CTL from precursor cells during the incubation time : In the present study, high density lymphocytes were found to be necessary for the observed augmentation of cytotoxic activity. Thus, the low-density lymphocytes alone kept in culture for 24 hr. showed a drastically decreased activity (Table I, Expt. No. 2). Generation of low-density CTL from high-density precursors during 24 hr. in mixed lymphocyte cultures, has been well established (MacDonald *et al*, 1974).

d) Pre-incubation procedure may remove blocking factors (antigen or antibody or immune complexes) from the surface of the lymphocytes. Blocking of specific receptors on the surface of lymphocytes by antigen or complexes, (Nind *et al*, 1973) and the release of these blocking factors by appropriate methods (Currie and Basham, 1972) have been shown previously. This explanation is more acceptable when we consider the experimental finding that the supernatant fluid from the pre-incubation mixture was able to abrogate the cytotoxicity of the fully active lymphocytes (Table II). It is interesting to note that the tumour immunity was seen to reach a peak on day 15 of the tumour intake and then rapidly declined afterwards (Fig. 1). It is easy to assume that the progressive tumour mass release large quantity of antigen molecules, which may block the recognition sites of the immune lymphocytes, leading to immune suppression *in vivo*. These antigen coating could be released from the lymphocytes during the incubation procedure, leading to the augmentation of *in vitro* cytotoxicity.

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Abbreviations used in this paper

T cells	=	Thymus-derived cells
B cells	=	Bone-marrow derived cells
CLT	=	Cytotoxic T lymphocytes
DMEM	=	Cytotoxic T lymphocytes
FCS	=	Foetal calf serum
BSA	=	Bovine serum albumin
Ig	=	Immunoglobulin

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