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Natural cytotoxic reactivity of human lymphocyte subpopulations

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Summary. The spontaneous cytotoxicity of human peripheral blood lymphocyte preparations from normal donors for K562 target cells was examined. Effector cells were separated into SRBC rosette forming cell (RFC) and non-rosette forming cell (non-RFC) fractions using optimal and suboptimal rosetting procedures. RFC and non-RFC fractions both had high cytotoxic activity irrespective of the rosetting procedure. Owing to the larger size of the RFC fraction, it contained a higher proportion of the total activity in the preparation. Nylon fibre column adherent and non-adherent fractions also both produced cytotoxicity. Nylon fibre non-adherent cells separated by SRBC separation gave a RFC fraction with low activity and a non-RFC fraction with high activity. Separation of nylon fibre adherent cells gave RFC and non-RFC fractions with high cytotoxic activity. Therefore cytotoxic cells did not form a discrete subpopulation and either occur in several lymphocyte subsets or show a variable capacity to form SRBC rosettes and adhere to nylon fibre.

INTRODUCTION

Human peripheral blood lymphocytes show spontaneous (natural) cytotoxicity (NC) towards a variety of allogeneic and xenogeneic tissue culture cell lines (Takasugi, Mickey & Terasaki, 1973; Kay & Sinkovics, 1974; Rosenberg *et al.*, 1974; Jondal & Pross, 1975; Oldham *et al.*, 1975; Pross & Jondal, 1975; Kiuchi & Takasugi, 1976). The human myeloid cell line K562 is particularly susceptible to natural cytotoxicity and measurement of ^{51}Cr release from these cells provides a sensitive and reproducible assay of this activity (Jondal & Pross, 1975; West, Cannon, Kay, Bonnard & Herberman, 1977a). Natural cytotoxicity is the property of a subpopulation of lymphocytes and may be used as a functional marker for this subpopulation (Jondal & Pross, 1975; Pross & Jondal, 1975; Kiuchi & Takasugi, 1976). There are conflicting reports on the surface marker characteristics of the cells responsible for the phenomenon in human peripheral blood, particularly concerning the expression of SRBC receptors and complement receptors (Jondal & Pross, 1975; Pross & Jondal, 1975; Kiuchi & Takasugi, 1976; West *et al.*, 1977a; DeVries, Cornain & Rümke, 1974; Pape, Troye & Perlmann, 1977; Vessella, Gormus, Lange & Kaplan, 1978), but there is virtual unanimity that the cells express receptors for the Fc portion of immunoglobulin G (Jondal & Pross, 1975; Kiuchi & Takasugi, 1976; West *et al.*, 1977a; Pape *et al.*, 1977; Bakács, Gergely, Cornain & Klein, 1977; Peter, Pavie-Fischer, Fridman, Aubert, Cesarini, Roubin & Kourilsky, 1975; Kay, Bonnard, West & Herberman, 1977; Nelson, Bundy & Strober, 1977).

We have examined some of the characteristics of the lymphocyte population with natural cytotoxicity for

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the K562 cell line using separated lymphocyte subpopulations. In particular, attention has been focused on the cytotoxicity produced by SRBC rosette forming and non-rosette forming populations, since it has been reported that the cytotoxic cell may express a low affinity receptor for SRBC (West *et al.*, 1977a). Human blood lymphocytes were rosetted with SRBC under a number of experimental conditions, reported to produce optimal or suboptimal rosette formation and separated by density gradient centrifugation to give rosette forming and non-rosette forming fractions. Rosettes were formed with SRBC pre-treated with neuraminidase or amino-ethylisothiouronium bromide hydrobromide (AET) and using a high SRBC-lymphocyte ratio (200:1), three procedures reported to enhance rosette formation (Galili & Schlesinger, 1974; Weiner, Bianco & Nussengweig, 1973; Kaplan & Clark, 1974; Pellegrino, Ferrone, Dierich & Reisfeld, 1975; West *et al.*, 1977a); and also using a 29° incubation period and no incubation period, two procedures reported to detect only cells with high affinity SRBC receptors (Wybran & Fudenberg, 1973; West, Sienknecht, Townes & Herberman, 1976; West *et al.*, 1977a). The effects on cytotoxic activity of the depletion of cells adherent to nylon fibre columns or plastic surfaces or the depletion of phagocytic cells was also examined. The results reported here indicate that cells with cytotoxic activity do not form a single subpopulation in terms of their ability to form rosettes with SRBC or to bind to nylon fibre columns.

MATERIALS AND METHODS

Lymphocyte preparation and depletion

Heparinized peripheral blood from healthy donors was initially separated by centrifugation on Ficoll–Triosil (F-T) (Potter & Moore, 1975). In some experiments, lymphocyte preparations were depleted of cells adhering to nylon fibre columns or plastic surfaces or depleted of cells phagocytosing carbonyl iron. The separation methods and composition of these populations have been described in detail previously (Potter & Moore, 1975; 1977). In some experiments, cells adhering to nylon fibre columns were recovered by vigorously teasing the nylon fibre in RPMI 1640 medium containing 10% foetal calf serum (RPMI-FCS).

SRBC rosette formation and separation

Rosette formation with SRBC was performed using a standard technique (Potter & Moore, 1975) (ST) and also various modifications described by others to give optimal or suboptimal rosette formation. The standard technique consisted of mixing SRBC and lymphocytes at a ratio of 40:1, pelleting the cells by centrifugation and incubating at 4° for at least 2 h.

The following modified techniques were also used.

Neuraminidase-treated SRBC (SRBC-neuramin). The SRBC used for rosetting were pre-treated with neuraminidase (BDH Neuraminidase, 12·5 units /ml/10^8 SRBC for 30 min at 37°).

AET-treated SRBC (SRBC-AET). The SRBC used for rosetting were pre-treated with AET (amino-ethylisothiouronium bromide hydrobromide) (1 vol. SRBC + 4 vol. 0·143 M AET, pH 9·0, 15 min, 37°).

200:1, SRBC:Lymphocyte ratio (SRBC-200:1). The SRBC:lymphocyte ratio used for rosetting was increased from 40:1 to 200:1.

Rosette formation at 29° (SRBC-29°). The incubation temperature was increased from 4 to 29°.

Early (active) rosette formation (SRBC-early). Rosettes were examined immediately after centrifugation with no incubation period.

Rosette forming cells were separated by centrifugation on F–T (Potter & Moore, 1975) and pellet and interface populations were collected. In preliminary experiments, the effect of lysing SRBC with Tris–NH_4Cl solution (0·75% NH_4Cl in Tris buffer pH 7·2) was examined, but in all subsequent experiments SRBC were lysed by treatment with distilled water for 5 s. The degree of separation achieved was examined by re-rosetting samples from the pellet and interface population using the standard SRBC rosetting method.

Cytotoxicity assay

The target cells used in this assay were the K562 human myeloid cell line (obtained from the Karolinska Institute, Stockholm) grown in suspension in RPMI-FCS. Target cells (3–5 × 10^6) were labelled for 90 min at 37° using 100 μCi sodium [^{51}Cr]-chromate (Radiochemical Centre, Amersham) and then washed four times in RPMI. Labelled target cells were re-suspended in RPMI-FCS and 0·2 ml samples containing 10^4 cells were added to 2·5 ml plastic tubes. Effector cells were added to the tubes in a further 0·2 ml volume

of RPMI-FCS and effector to target ratios of 40:1, 20:1, 10:1 and 5:1 were used routinely. Control tubes containing target cells only were included to give the background isotope release and maximum release was determined by adding Triton X100 (1/100 dilution) to target cells. All tests were set up in triplicate and incubated for 18 h at 37° in an atmosphere of 95% air and 5% CO_2. At the end of the incubation period the tubes were spun at 1200 rpm for 10 min, 0·2 ml samples samples of the supernatant were removed from each tube and both aliquots counted on a Searle 1185 gamma counter. The percentage ^{51}Cr release was determined for each tube and using the mean value of the triplicate tubes the percentage cytotoxicity was calculated according to the following formula:

$$\text{percentage cytotoxicity} = \frac{(\%\ ^{51}\text{Cr release in sample} - \%\ ^{51}\text{Cr release in control})}{(\%\ ^{51}\text{Cr release in Triton} - \%\ ^{51}\text{Cr release in control})} \times 100$$

The background isotope release was 15–25% and the maximum release 85–98%.

All experiments were repeated on at least four separate occasions and unless otherwise stated results represent the mean values from four experiments expressed as percentage cytotoxicity (±SE). Results are also expressed in terms of lytic units/10^7 effector cells (one lytic unit being defined as the number of effector cells required to produce 35% cytotoxicity above the base line, determined from a dose response curve for the effector cell population). In some experiments where the effector population was split into a rosette forming and non-rosette forming fraction the results are also expressed in terms of the percentage of the total recovered cytotoxic activity that is present in each fraction (by determining lytic units/10^7 cells and then lytic units/fraction).

RESULTS

Ficoll–Triosil lymphocyte preparations from normal donors consistently produced high levels of cytotoxicity against K562 target cells. In forty-two tests, the mean value for cytotoxicity at a 40:1 effector to target cell ratio was 63% (with a range of values from 50 to 78%) and at a ratio of only 5:1, the mean cytotoxicity was still 32%.

The cytotoxic activity of Ficoll–Triosil lymphocyte preparations was examined following depletion of adherent and phagocytic cells. Removal of cells adhering to plastic surfaces or cells ingesting carbonyl iron resulted in a slight increase in cytotoxicity and removal of cells adhering to nylon fibre columns did not alter the level of cytotoxicity produced (Table 1).

In preliminary experiments the effect of treating lymphocytes with Tris-buffered NH_4Cl (Tris–NH_4Cl) solution or distilled water to lyse SRBC added for rosette formation was examined. Lymphocyte preparations were mixed with SRBC as for rosette formation and then subjected to Tris-NH_4Cl or H_2O lysis. Lymphocyte preparations treated with Tris–NH_4Cl had a lower level of cytotoxicity than control untreated cells and cells treated with H_2O which had similar activity (Fig. 1). We have also found reduced cytotoxicity following Tris–NH_4Cl treatment of rat lymphocytes (Potter & Moore, 1978). In subsequent experiments in which cells were recovered after rosette sedimentation pellet and interface populations were treated with H_2O to remove SRBC.

Table 1. Percentage cytotoxicity of K562 target cells by blood lymphocyte preparations depleted by nylon column filtration, plastic adherence or carbonyl iron uptake

	Effector:target cell ratio			
Population	40:1	20:1	10:1	5:1
F/T	55·6±4·4	46·3±2·4	32·7±4·3	23·1±5·7
F/T+plastic	62·1±5·5	53·5±5·7	41·9±6·6	29·9±7·5
F/T+carbonyl iron	69·8±6·8	60·3±5·1	45·1±7·2	35·3±5·8
F/T+nylon column	53·8±6·9	47·1±7·0	37·3±5·3	23·1±4·4

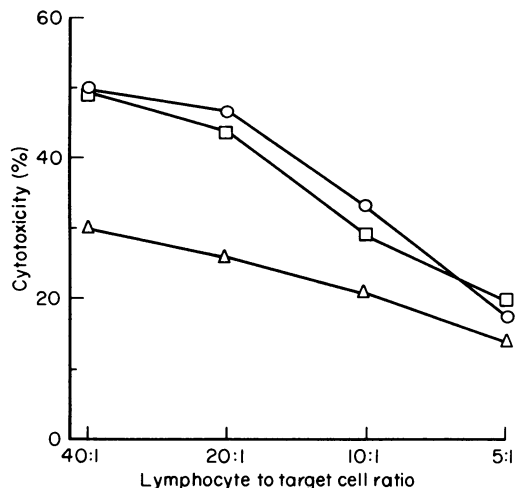

Figure 1. The effect of erythrocyte lysis on the spontaneous cytoxicity of blood lymphocytes for K562 target cells. Lymphocyte preparations were mixed with SRBC and then subjected to Tris–NH_4Cl (△) or distilled water (□) erythrocyte lysis; results are compared with those for control untreated cells (○).

Lymphocyte preparations were rosetted with SRBC and separated into a pellet population enriched with rosette forming cells (RFC) and an interface population enriched with non-rosette forming cells (NRFC). These two populations, together with control unseparated cells were tested for cytoxocity against K562 target cells. The mean results for fourteen experiments are shown in Table 2. The interface population depleted of RFC gave slightly higher percentage cytotoxicity than the control population (average values at 40:1 ratio, 64 and 61% respectively). The pellet population gave on average lower levels of cytotoxicity than the control and interface population (51% at 40:1 ratio) and the results were more variable. The pellet and interface populations differed widely in their composition, containing on average 89 and 7% SRBC-RFC respectively when re-rosetted, but both populations still produced considerable cytotoxic activity. Very similar results were obtained when Ficoll–Paque was used for lymphocyte separation and SRBC rosette separation in place of Ficoll–Triosil.

The effect of depleting lymphocyte preparations of adherent and phagocytic cells before SRBC rosette separation was also examined. The results for control, pellet and interface populations from lymphocyte preparations depleted by plastic adherence and carbonyl iron uptake showed a very similar pattern to the results obtained without depletion, but with slightly higher activity (Table 3). The interface population gave higher activity than the control (75 and 70% respectively at 40:1 ratio) and the pellet lower activity (53% at 40:1) but again all three populations were consistently active.

To further examine the cytotoxic activity of the SRBC-RFC population separations were performed on cells rosetted under a variety of conditions reported to enhance or reduce rosette formation. Pellet and interface populations from cells rosetted at 29° or with no incubation period revealed similar levels of cytotoxicity to populations from conventional rosette separations despite the fact that a lower precentage of cells formed rosettes under these conditions (58 and 35% RFC respectively) (Table 4). When cells were separated following rosette formation using neuraminidase or AET treated SRBC or at a higher SRBC-lymphocyte ratio, the RFC population gave slightly higher cytotoxicity than conventional RFC. Rosette separation performed using the six procedures did not produce any major changes in the cytotoxic activity of rosette forming and non-rosette forming populations; in all cases the interface population had greater activity than the pellet, but the pellet populations always

Table 2. Percentage cytotoxicity of K562 cells by blood lymphocyte preparations separated by SRBC rosette formation.

Population	E rosettes* (%)	Effector:target cell ratio 40:1	20:1	10:1	5:1
Control	66·5±1·3	61·2±2·5	50·0±2·4	38·9±4·1	30·4±3·3
Pellet	89·3±1·0	50·8±4·6	41·5±4·4	31·9±4·6	23·3±3·4
Interface	6·6±1·2	64·3±2·6	52·6±3·3	40·2±3·0	31·2±3·2

* Percentage SRBC rosettes when re-rosetted

Table 3. Percentage cytotoxicity of K562 target cells by blood lymphocyte preparations depleted by plastic adherence and carbonyl iron uptake before SRBC rosette separation

Population	E rosettes* (%)	Effector : target cell ratio 40:1	20:1	10:1	5:1
Control	69·7±1·6	69·9±3·7	58·3±4·7	49·4±7·7	39·7±5·7
Pellet	89·0±1·6	53·1±6·5	46·6±4·8	39·0±5·3	30·7±4·5
Interface	6·4±3·2	75·1±2·9	63·7±4·0	57·4±2·2	40·0±3·7

* Percentage SRBC rosettes when re-rosetted

Table 4. Percentage cytotoxicity of K562 target cells by blood lymphocyte preparations separated by modified SRBC rosette procedures

				Cytotoxicity (%)§							
		E rosettes (%)‡		Pellet				Interface			
Rosetting procedure*	%RFC†	Pellet	Interface	40:1	20:1	10:1	5:1	40:1	20:1	10:1	5:1
SRBC	69·5	87·0	8·2	53·1	47·6	40·6	29·0	67·0	59·9	51·9	36·7
SRBC-29°	57·8	89·0	14·7	51·1	43·5	33·0	20·6	70·9	58·9	50·6	32·1
SRBC-early	34·5	82·8	25·2	54·5	53·8	42·1	31·2	63·0	51·4	36·6	26·8
SRBC-neuramin	73·4	91·5	7·6	66·0	56·7	44·9	32·4	67·6	60·0	48·9	37·8
SRBC-AET	71·2	90·2	5·4	58·6	49·5	39·1	32·5	58·8	49·9	37·3	30·6
SRBC-200:1	70·5	87·7	7·9	58·2	52·6	42·8	33·9	64·5	56·3	48·0	34·1

* Rosetting procedures as indicated in Materials and Methods.
† Percentage RFC using modified procedures.
‡ Percentage SRBC rosettes when rerosetted with untreated SRBC.
§ Percentage cytotoxicity at effector : target cell ratios shown.

retained a high level of cytotoxicity (>51% at 40:1 ratio).

These results were obtained using the same effector to target cell ratios (40:1 to 5:1) for each cell population, but since the separated populations were of unequal size the percentage cytotoxicity was not representative of the total cytotoxic activity of each population. The results were also expressed in terms of the percentage of the total recovered activity that was present in each fraction (Table 5). In this case cells rosetted under conventional or enhancing conditions gave pellet populations containing 60–74% of the activity and interface populations with 26–40% of the activity. Suboptimal rosette formation gave pellet populations with 41–42% of the activity and interface populations with 58–59% of the activity. Rosette formation under optimal and suboptimal conditions therefore resulted in changes in the size of the rosette forming population but not the cytotoxic activity on a cell to cell basis, which remained similar to the activity of the non-rosette forming population.

Standard SRBC rosette separation was also performed on lymphocyte preparations that were depleted by nylon fibre column filtration. The cytotoxic activity of pellet and interface fractions and unseparated cells from depleted and non-depleted populations were compared. The interface fractions from depleted and non-depleted populations both produced cytotoxicity equal to or greater than control unseparated cells (Fig. 2). The pellet fraction from the depleted population gave considerably less cytotoxicity than the corresponding fraction from the non-depleted population

Table 5. Cytotoxicity of K562 target cells by blood lymphocyte preparations separated by modified SRBC rosette procedures expressed as lytic units and recovered activity per fraction.

Rosetting procedure*	Lytic units/10^7 cells†		Recovered activity (%)‡	
	Pellet	Interface	Pellet	Interface
SRBC	130	204	60	40
SRBC-29°	83	167	41	59
SRBC-early	147	108	42	58
SRBC-neuramin	161	213	67	33
SRBC-AET	141	120	74	26
SRBC-200:1	175	189	68	32

* Rosetting procedures as indicated in Materials and Methods.
† Lytic units/10^7 effector cells required for 35% cytotoxicity.
‡ Percentage total recovered lytic units present in each fraction.

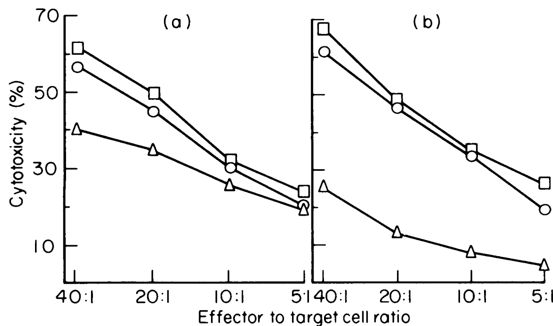

Figure 2. Cytotoxicity of K562 target cells by blood lymphocyte preparations separated by (a) SRBC separation and (b) nylon fibre column filtration and SRBC rosette formation. Nylon fibre column filtered and untreated cells were separated into pellet (RFC) (△), interface (non-RFC) (□) and control unseparated (○) populations.

(average value 25 and 40% respectively at 40:1 ratio) despite containing similar numbers of RFC (89%).

The cytotoxic activity of nylon column adherent cells was examined by recovering cells that could be removed from the nylon fibre by vigorous teasing. This population which represented approximately 30% of the initial preparation produced high levels of cytotoxicity but less than the non-adherent population with average values of 40 and 60% respectively at a ratio of 40:1 (Table 6). When nylon column adherent cells were fractionated into SRBC RFC and non-RFC fractions both were found to be cytotoxic (38 and 49% cytotoxicity respectively at 40:1 ratio) (Table 6). Therefore cytotoxic activity was found in both nylon fibre adherent and non-adherent populations and the activity in the non-adherent population was greatest in the non-RFC fraction while in the adherent population activity was high in RFC and non-RFC fractions.

DISCUSSION

Human peripheral blood lymphocyte preparations were found to express high levels of spontaneous cytotoxicity against K562 target cells as previously described by others (Jondal & Pross, 1975; West *et al.*, 1977a). Cytotoxicity activity was not reduced by removal of cells adherent to nylon fibre columns or plastic surfaces or of cells which ingested carbonyl iron. Since nylon column filtration depletes B lymphocytes as well as non-lymphocytes in the preparation (Potter & Moore, 1977) these results are in agreement with other reports indicating that human blood contains a non-B lymphocyte which has spontaneous cytotoxic activity against tissue culture cell lines (West *et al.*, 1977a; Nelson *et al.*, 1977). Cytotoxicity against tissue culture cell lines has also been reported as being the property of non-T lymphocytes (Pross & Jondal,

Table 6. Percentage cytotoxicity of K562 target cells by nylon column adherent and non-adherent blood lymphocyte preparations fractionated by SRBC rosette separation.

SRBC Separation	Nylon column non-adherent effector:target cell ratio				Nylon column adherent effector:target cell ratio			
	40:1	20:1	10:1	5:1	40:1	20:1	10:1	5:1
Control	59·8±6·1	48·1±8·1	36·6±7·0	22·3±5·1	40·2±6·4	32·3±8·2	25·8±5·3	17·2±3·8
Pellet	24·5±2·9	12·8±2·8	7·1±0·8	4·0±1·4	37·8±2·5	25·7±3·0	19·8±3·5	10·2±2·0
Interface	69·4±3·8	53·5±6·9	38·8±6·1	30·6±5·4	48·7±3·9	39·2±2·7	28·5±4·7	17·1±5·6

1975; DeVries *et al.*, 1974) or non-T non-B lymphocytes (Kiuchi & Takasugi, 1976; Bakács *et al.*, 1977) suggesting that the cytotoxic cell does not have the surface marker characteristics of T cells or B cells. The cytotoxic population has been found in many cases to contain Fc receptor-bearing cells (Jondal & Pross, 1975; Kiuchi & Takasugi, 1976; West *et al.*, 1977a; Pape *et al.*, 1977; Bakács *et al.*, 1977; Peter *et al.*, 1975; Kay *et al.*, 1977; Nelson *et al.*, 1977) so activity does not correspond to a true null cell population. Spontaneous cytotoxicity has also been found by some workers to be present in the SRBC RFC population (West *et al.*, 1977a; Kay *et al.*, 1977; Saksela, Hayrey & Andersson, 1977).

In the present study, cytotoxic activity was found in the SRBC-RFC fraction, but on a cell to cell basis greater activity was present in the non-RFC fraction. Since the RFC fraction represented approximately 70% of the population, it contained a greater proportion of the total activity recovered following separation. These results concur with those of Kay *et al.* (1977) who also used SRBC separation and K562 target cells.

Modification of the rosetting procedure to give enhanced or suboptimal rosette formation did not significantly change the cytotoxic activity of the RFC fraction when tested using equal numbers of cells but the size of the RFC fraction was altered. Even using enhanced rosetting procedures, separation of the RFC fraction did not produce enrichment of cytotoxic activity on a cell to cell basis. The results clearly demonstrate the cytotoxic activity was present in both RFC and non-RFC fractions from SRBC rosette separations and that the cytotoxic cells were not exclusively SRBC receptor positive or negative.

West *et al.* (1977a) also observed cytotoxic activity against K562 target cells in SRBC RFC fractions and suggested that cells with low affinity SRBC receptors were responsible since RFC fractions from 29° rosettes had considerably less activity. In our experiments however, suboptimal rosette formation (using early and 29° rosettes) produced RFC fractions which still contained high levels of activity demonstrating that RFC fractions from both optimal and suboptimal separations had comparable activity. Saksela *et al.* (1977) have also found high levels of cytotoxicity against K562 in RFC fractions from both optimal and early rosette separations. Using a human lymphoblastoid target cell line Eremin, Coombs, Plumb & Ashby (1978) obtained similar cytotoxicity with unseparated blood lymphocyte populations and T cells prepared by SRBC rosette separation, but T cells prepared by depleting Fc and C3 receptor-bearing cells had lower activity. They suggest that the activity in the SRBC pellet population was due to migration of Fc receptor bearing non-T lymphocytes into the pellet along with RFC. Since human lymphocytes expressing receptors for both SRBC and the Fc portion of IgG have been described (Kay *et al.*, 1977; Ferrarini, Moretta, Abrile & Durante, 1975; Moretta, Ferrarini, Mingari, Moretta & Webb, 1976; West, Payne, Weese & Herberman, 1977b; Gupta & Good, 1977) this subopulation of cells may be responsible for activity in the SRBC pellet population.

The cytotoxic activity of the RFC formation was reduced if the lymphocyte preparation was passed through a nylon fibre column before SRBC rosette separation, but not if depleted by plastic adherence and carbonyl iron uptake. Much of the activity in the RFC fraction therefore appeared to be in the nylon fibre adherent population. Bakács *et al.* (1977) also found that nylon fibre column filtration before SRBC rosette separation reduced the cytotoxic activity of the RFC fraction using tumour monolayer target cells. It is clear therefore, that the cytotoxic activity of the SRBC RFC fraction is greatly influenced by the lymphocyte isolation procedures that are used before SRBC separation and this may account for some of the differences found in the activity of RFC fractions.

When nylon fibre adherent cells were recovered this population was found to contain considerable cytotoxic activity and when separated the activity was present in both RFC and non-RFC fractions.

Cells with spontaneous cytotoxic activity for K562 target cells were therefore found to be present in nylon fibre adherent and non-adherent fractions and SRBC RFC and non-RFC fractions. Cytotoxic cells did not form a discrete subpopulation and it appears that either several subpopulations express this activity or the cytotoxic cells show a variable capacity to adhere to nylon fibre and form SRBC rosettes. It is not clear from the present study if the cytotoxic cells in the RFC fraction are true T lymphocytes or a subpopulation of non-T cells which also rosette with SRBC.

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