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A Novel Nonphagocytic Mechanism of Erythrocyte Destruction Involving Direct Cell-Mediated Cytotoxicity

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Abstract

The number of erythrocytes fell when co-cultured with cell preparations derived from mouse spleen, thymus, bone marrow, or peritoneal exudate (PE) cells. Erythrocyte-depletion activities (EDA) of different leukocyte preparations were in the order PE > spleen > thymus > bone marrow. Adherent, nonadherent, T-depleted, and T-enriched cell subpopulations had comparable EDA. Spleen cells from athymic nude mice, however, lacked significant EDA. In addition, EDA was boosted by Concanavalin A (Con A) but not by lipopolysaccharide, indicating that T cells may play a crucial role in inducing EDA in spleen cells. Paraformaldehyde-fixed spleen or PE cells, as well as membrane preparations isolated from spleen cells, efficiently lysed erythrocytes. Erythrocyte ghost membranes inhibited erythrocyte lysis by control or paraformaldehyde-fixed spleen cells. Treatment with hamster anti-mouse Fas or anti-mouse tumor necrosis factor receptor (TNFR) antibody could opsonize erythrocytes for faster depletion by spleen cells, suggesting an expression of Fas and TNFR on erythrocytes. TNFα could lyse erythrocytes in a dose-dependent fashion. Additionally, enhanced spleen cell EDA induced in response to succenyl Con-A could be blocked by anti-TNFα antibodies. Our results provide evidence for a direct cell-mediated cytotoxicity (CMC) of erythrocytes by leukocytes. A role of molecules of Fas and the TNF family in CMC of erythrocytes has also been suggested. Further work is needed to understand if, and to what extent, CMC of erythrocytes contributes to erythrocyte destruction in vivo and to determine its patho-physiological significance. *Int J Hematol.* 2000;71:227-237

Key words: Erythrocytes; cytolysis; tumor necrosis factor; Fas; erythrophagocytosis

1. Introduction

The human body has 3 to 5×10^{13} erythrocytes in circulation. It is estimated that the average life span of erythrocytes is about 100 days (50 days in rodents) [1,2]. About 1% of all erythrocytes (2% in rodents) must therefore be killed each day, and in order to sustain the levels of circulating erythrocytes, an equal number must be added daily to the circulation. Processes of erythropoiesis and erythrocyte destruction must be finely balanced to ensure that the required number of erythrocytes stay in circulation, and they must be flexible enough to meet contingencies arising from sudden loss of blood or increased demand in oxygen-carrying capacity in hypoxic conditions. Numbers of erythrocytes can change and remain high (polycythemia) or low (anemia), and these shifts must be accounted for by altered rates of erythrocyte generation and/or destruction. In order to understand the homeostasis of erythrocytes, mechanisms involved in their generation and destruction must be clearly understood. Although a great deal is known about the process of erythropoiesis and the role played by a variety of cytokines and growth factors in this process [3-6], the mechanisms involved in the destruction of erythrocytes are not well understood. It is believed that aging erythrocytes are trapped in the spleen and bone marrow, where phagocytic cells of the reticuloendothelial system remove these cells [7,8]. How aging erythrocytes are recognized by the phagocytes is not clear, although there are several speculations in the literature about the process of senescence of erythrocytes and the changes that may lead to their recognition by the reticuloendothelial system [9-12]. Opsonization of senescent erythrocytes by

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antibodies and complements to facilitate their phagocytosis has also been suggested [13,14].

Although erythrocyte turnover has been accurately assessed in vivo [1,2], elucidation of various mechanisms directly involved in erythrocyte destruction in vivo are not fully understood. It is also not clear if mechanisms other than phagocytosis participate in erythrocyte destruction in vivo. In the present study, we have used an in vitro co-culture system to examine the mechanisms of erythrocyte destruction by leukocytes. Evidence has been obtained for direct cell-mediated cytotoxicity (CMC) of erythrocytes by leukocytes, which, to our knowledge, has not been demonstrated so far. In addition, our results also suggest that the tumor necrosis factor (TNF) family of molecules may be involved in the mechanism of erythrocyte lysis by leukocytes.

2. Materials and Methods

2.1. Animals

Inbred female C57Bl/6 mice (8 to 12 weeks old) used in this study were bred and maintained in the animal house facility in Jawaharlal Nehru University, New Delhi, India. Nude (nu/nu) mice were obtained from the National Institute of Nutrition (NIN), Hyderabad, India.

2.2. Culture Medium

Unless otherwise specified, all the culture work was done in RPMI-1640 from Sigma (St. Louis, MO, USA), supplemented with 10% fetal calf serum (FCS), 2×10^{-5} M 2-mercaptoethanol (ME), 300 μg/mL glutamine, and 60 μg/mL gentamicin (complete medium or CM).

2.3. Reagents and Other Supplies

Special reagents used in this study and their sources are as follows. Lympholyte M (Cedarlane Laboratories, Canada); Paraformaldehyde (S. D. Fine CHEM, India); Carbonyl iron, Concanavalin A (Con A), succenyl Con-A (SCA), and lipopolysaccharide (LPS) (Sigma); purified hamster anti-mouse Fas monoclonal antibody (PharMingen, San Diego, CA, USA); purified anti-TNF receptor antibody (Genzyme, Cambridge, MA, USA); hamster immunoglobulin (IgG; Organon Teknika, West Chester, UK); TNFα, anti-TNFα antibody (R&D Systems, Minneapolis, MN, USA); DCCM2 serum-free culture medium (Biological Industries, Oshrat, Israel); and all plastic disposable culture ware (Corning Costar, Bodenheim, Germany).

2.4. Effector Cells

Isolation of various mouse leukocyte preparations has been described before [15]. To get erythrocyte-free spleen cells, erythrocytes were removed by hypotonic shock. Spleen cells were pelleted (1800 rpm × 5 minutes), and 500 μL of sterile water was added to the pellet drop by drop and the tube was vortexed for 15 seconds, followed immediately by the addition of an equal volume of 2× phosphate buffered saline (PBS). The effector cells were fixed by adding 500 μL of 1% paraformaldehyde to loosened the cell pellet. Cells were washed 4 times with medium (RPMI-1640 + 2% FCS) and suspended at desired concentrations in CM.

2.5. Target Cells

Mouse blood was collected aseptically by eye bleed. The blood was collected into a tube containing sterile PBS. The cells were washed thrice using sterile PBS. The erythrocyte pellet was then suspended in CM. These cell preparations were >99.9% erythrocytes, and were used as such. In some experiments, a purer preparation of erythrocytes was obtained by layering the erythrocyte suspension on 500 μL of Lympholyte M, followed by centrifugation at 1500 rpm for 20 minutes. Pelleted erythrocytes were washed 4 times with RPMI-1640 medium containing 2% FCS and suspended in CM at the desired concentration.

2.6. Fractionation of Mouse Spleen Cells

Spleen cell preparations were depleted of adherent and phagocytic cells by plastic adherence and carbonyl iron and magnet treatment, or enriched for T cells by passage through a nylon wool column (NWC), as described previously [15]. NWC-passed cells were >75% positive for CD3, as assessed by flow cytometry. For depleting T cells by anti-Thy-1 + C treatment [16], 120×10^6 spleen cells were suspended in 6 mL of 10× concentrated supernatant of hybridoma TIB99 (anti-Thy-1 antibody) and incubated on ice for 30 minutes. Rabbit serum in the amount of 1.8 mL was added as the source of complement, and incubation proceeded at 37°C for 1 hour. Cells were washed 3 times and suspended in CM. By flow cytometry, T-depleted preparations had fewer than 2% CD3-positive cells.

2.7. Preparation of Spleen Cell Membrane

Spleen cells (25×10^6) were suspended in buffer A (0.01 M tris HCl, pH 7.4, 0.001 M $MgCl_2$, 0.001M KCl) and frozen at –70°C for 15 minutes. The suspension was frozen and thawed repeatedly (5 to 6 times) to break the cells. Cell suspensions were checked under the microscope to ensure that all the cells were lysed. The pH of the suspension was adjusted to 8.0 with tris base, and the suspension was stirred at 4°C for 10 minutes. The suspension was then centrifuged at 1500 *g* for 5 minutes, and supernatant was collected in a sterile tube. The pellet was washed repeatedly with buffer B (0.15 M NaCl, 0.01 M tris HCl, pH 8.0) until the supernatant was clear. The combined supernatant was centrifuged at 2000 g for 10 minutes to remove the debris and then at 105,000 g for 1 hour to pellet the membranes. The membrane was suspended in sterile PBS or RPMI-1640 (plain medium) and kept frozen at –70°C.

2.8. Preparation of Erythrocyte Ghosts

To erythrocyte pellets (30×10^6 cells), 25 mL of NaH_2PO_4 was added (10 mM NaH_2PO_4; pH was adjusted to 7.4 by 1N NaOH). The suspension was centrifuged at 20,000 g for 40 minutes. The process was repeated 3 to 4 times until the super-

natant was colorless or a faint pink. The pellet was again washed in PBS, and erythrocyte ghosts were suspended in CM.

2.9. Determination of EDA of Different Effector Agents

Erythrocytes (5×10^6/mL) were co-cultured with different types of leukocytes, membranes, or TNF at indicated concentrations for up to 3 days at 37°C in a CO_2 incubator. At different time points, residual erythrocytes in culture were estimated by counting in a hemocytometer. Assay wells were generally set in duplicates or triplicates. For each assay well, at least 200 erythrocytes were counted in the hemocytometer, and variations within replicates were generally within 5%. For the sake of clarity, mean counts of surviving erythrocytes have been plotted as percentage of erythrocytes added in the beginning of the culture (5×10^6/mL until otherwise mentioned). Each experiment was done 4 to 6 times, and representative data have been shown in each case.

2.10. Chromium Release Assay of Cytotoxicity

Erythrocytes were labeled with sodium chromate (Cr^{51}) for 1 hour, washed 3 times, and suspended in CM (10×10^6/mL). Effector cells at desired concentrations were distributed in 96-well microtest plates (0.1 mL/well). Labeled erythrocytes were added (0.1 mL/well), and plates were centrifuged (400 rpm × 5 minutes) and incubated in a CO_2 incubator for 1 or 2 days. Spontaneous release of chromium from erythrocytes was 30% to 40% on day 1 and 40% to 50% on day 2. Maximum release of chromium by water lysis was about 95%. Calculation of lysis from chromium-release data was done as before [17].

3. Results

3.1. Depletion of Erythrocytes Co-Cultured With Leukocyte Preparations

Effect of leukocytes derived from different lymphoid organs on the survival of erythrocytes in vitro was studied. Erythrocyte-free cell preparations from spleen, thymus, bone marrow, and peritoneal lavage were co-cultured with blood-derived erythrocytes for 3 days. The numbers of erythrocytes that survived on days 1, 2, and 3 of the culture were assessed by counting in a hemocytometer. Results of a typical experiment are shown in Figure 1. About 80% of the erythrocytes survived when cultured alone for 3 days. Co-culturing with different leukocyte preparations resulted in a loss of erythrocytes. Peritoneal exudate (PE) cells were most efficient in removing erythrocytes (no erythrocytes survived in this case, even on second day). In general, the erythrocyte-depletion activity (EDA) of different leukocyte preparations was in the following order: PE > spleen > thymus > bone marrow.

3.2. EDA of Spleen Cell Subpopulations

Because phagocytosis is believed to be the primary mechanism of erythrocyte destruction, EDA associated with leukocytes could be due to the presence of phagocytic cells like macrophages in the effector cell preparations. Removal

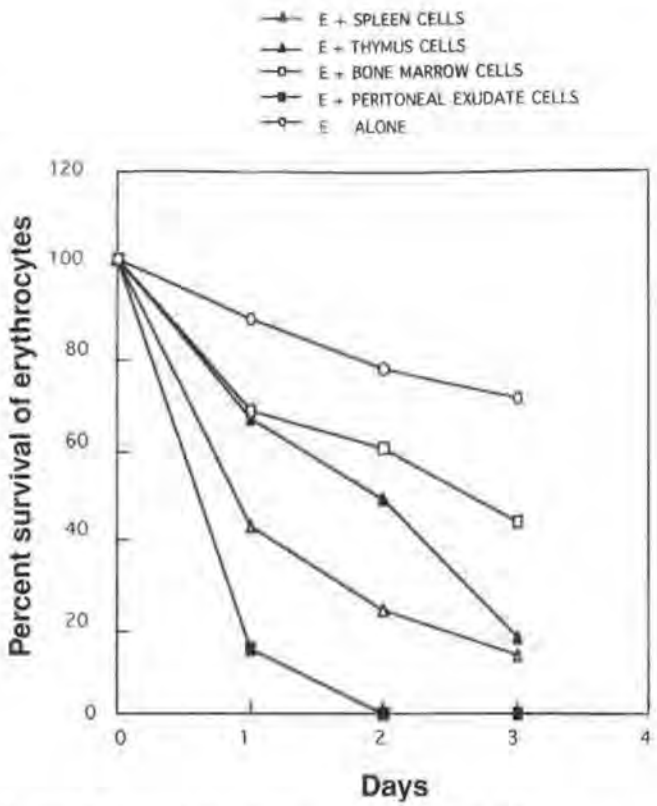

Figure 1. Survival of erythrocytes co-cultured with different preparations of leukocytes. C57Bl/6 erythrocytes (5×10^6/mL) were mixed with an equal number of cells derived from erythrocyte-depleted spleen, bone marrow, thymocytes, and peritoneal exudate cells and cultured in complete medium for 3 days. The surviving erythrocytes were determined on days 1, 2, and 3 of the culture. Each point on the graph represents the mean of 2 replicate observations.

of macrophages and other phagocytic cells should, in that case, lower the EDA of effector cell preparations. Results in Figure 2A, however, show that the EDA of adherent spleen cells (enriched in macrophages) was not higher than that of unfractionated spleen cells. In addition, plastic nonadherent spleen cells, further depleted of phagocytic cells by CoFe and magnet treatment (>90% depletion), not only retained their EDA but were more efficient than unfractionated spleen cells on the second day of culture (Figure 2B). In these experiments, enrichment or depletion of macrophages was confirmed by direct morphological examination, as well as by esterase staining criteria.

Spleen cells passed through an NWC (enriched for T cells) were less efficient than unfractionated spleen cells (Figure 2C). Depletion of T cells by anti-Thy-1 + C treatment had no effect on EDA (Figure 2D). These results suggest that adherent, nonadherent, T-depleted, and T-enriched subpopulations may all contribute to the EDA of spleen cells. Interestingly, however, spleen cells from athymic nude mice had virtually no EDA (Figure 3), indicating that T cells may be required for the generation of EDA in spleen cells, even though they are unlikely to be exclusive mediators of erythrocyte depletion.

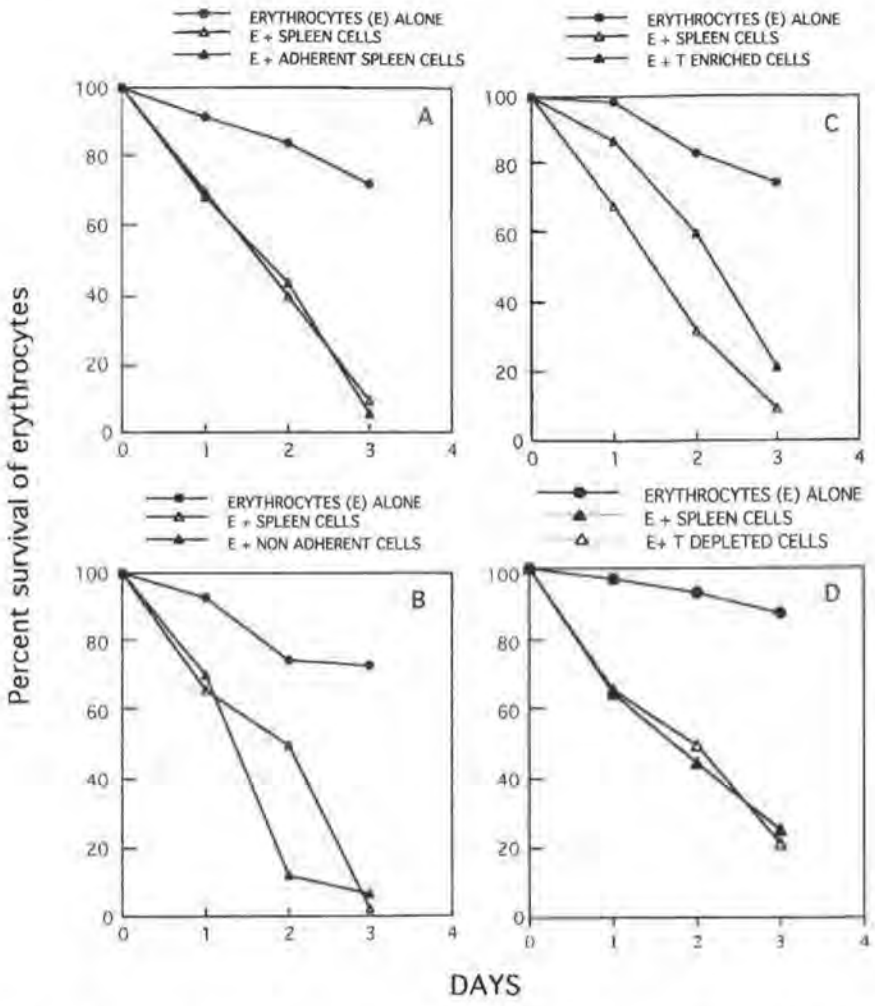

Figure 2. Effect of co-culturing with control, plastic adherent, plastic nonadherent, T-enriched, and T-depleted spleen cells on the survival of erythrocytes. Erythrocytes from C57Bl/6 mice (5×10^6 cells/mL) were cultured in complete medium with or without equal numbers of effector cells (unfractionated or plastic adherent spleen cells [panel A], nonadherent spleen cells [panel B], T-enriched spleen cells passed through a nylon wool column [panel C], or anti-Thy 1 + C–treated spleen cells [panel D]). Surviving erythrocytes in culture were determined on days 1, 2, and 3 of the culture. Each point on the graph depicts a mean of 2 replicate culture wells.

3.3. Augmentation of EDA by T-Cell Mitogens

Addition of T-cell mitogen Con-A resulted in a marked boosting of EDA in spleen-, thymus-, and bone marrow–derived cells, whereas B-cell mitogen LPS did not activate EDA in spleen cells (Figure 4). Con-A, being a tetrameric molecule with 4 carbohydrate binding sites, is known to agglutinate cells. The effect of Con-A on EDA was, however, not attributable to a bridging of effector and target cells, because SCA, a monomeric form of Con-A, had effects similar to tetrameric Con-A (results not shown). These results also suggest that T cells may have a role in inducing EDA.

3.4. Evidence for the Involvement of a Nonsecretory Pathway of Cytotoxicity in Killing of Erythrocytes

Results so far suggested that nonphagocytic effector cells may contribute to the EDA of spleen cells. The secretion of erythrolytic factors by control or Con-A–activated spleen cells, as a possible mechanism of EDA, was considered. Culture supernatants from control or Con-A–activated spleen cell preparations had no erythrolytic effect (results not shown). This finding indicated that control or mitogen-activated spleen cells did not release any erythrolytic mediator. Some soluble mediators of lysis, like cytolysin and its complements, require the presence of calcium ions for mediating their effect [18,19]. EDTA at a concentration that chelates all free calcium in the culture medium had no protective effect on erythrocytes, indicating that calcium ions were not required for erythrolytic effect of spleen cells (Table 1). In these experiments, a serum-free culture medium was used because the presence of serum would add an unspecified amount of calcium ions to the medium. The possibility of some other soluble erythrolytic mediator(s) was also examined by using the culture system in which spleen effector cells and erythrocytes were separated by semipermeable

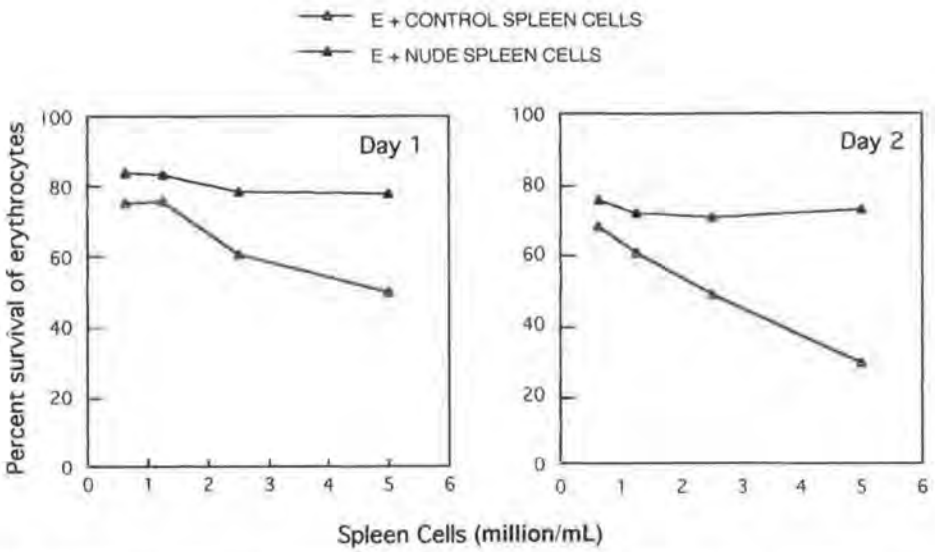

Figure 3. Dose response of the erythrocidal effect of spleen cells from euthymic and athymic mice. Erythrocytes (5×10^6 cells/mL) were co-cultured with different given concentrations of spleen cells from euthymic or nude athymic mice. The number of surviving erythrocytes was determined on day 1 and 2 of culture. Each point on the graph represents the mean of 2 replicate culture wells.

membranes (Costar Transwells). Also in these experiments, no evidence of erythrocyte killing by spleen cells separated by semipermeable membranes was obtained (results not shown). These results negate the possibility of involvement of soluble factors mediating erythrolytic effect and suggest that a direct cell-to-cell interaction may be needed for erythrolysis.

Results in Figure 5 show that fixing of effector cell preparations by paraformaldehyde did not abrogate the EDA of spleen cells. Indeed, spleen EDA was significantly enhanced if the effector cells were paraformaldehyde fixed. Fixing of spleen cells from athymic nude mice had no effect on their EDA, which remained very low (results not shown). The fixing of peritoneal exudate cells, which would abrogate their phagocytic activity, reduced but did not abolish their EDA (Figure 5). Similar results were obtained if other fixatives were used, such as glutaraldehyde and formaldehyde (results not shown). Lysis of erythrocytes by paraformaldehyde-fixed spleen cells was also examined by chromium-release assay of

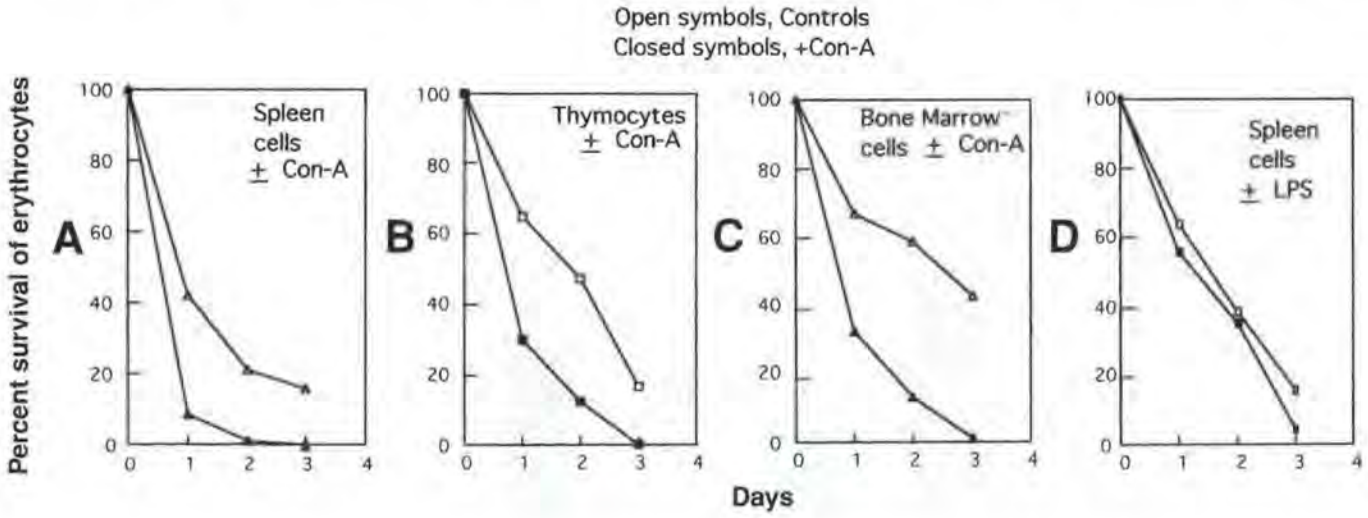

Figure 4. Clearance of erythrocytes from cultures of control and Con A–treated spleen, thymus, bone marrow, and peritoneal exudate cells from C57Bl/6 mice. A mixture of effector cell preparations derived from spleen, thymus, and bone marrow incubated with erythrocytes from C57Bl/6 mice (1:1 ratio, 5×10^6 cells/mL each) was cultured with or without Con A for 3 days (Panels A, B, and C). Spleen cells were also cultured with erythrocytes in the presence or absence of lipopolysaccharide (Panel D). The number of surviving erythrocytes was determined after 1, 2, and 3 days of culture. Each point on the graph represents the mean of 2 replicate culture wells.

Table 1.
Effect of EDTA on the Survival of Erythrocytes*

Cultures	EDTA (mM)	Percentage Survival of Erythrocytes		
		Day 1	Day 2	Day 3
Erythrocytes alone	None	92.4	84.4	69.9
Do	1.0	95.3	84.8	66.8
Do	2.0	94.3	85.7	54.0
Do	5.0	92.8	79.0	50.0
Erythrocytes + spleen cells	None	66.6	43.8	19.2
Do	1.0	54.3	43.8	21.0
Do	2.0	56.1	44.7	21.4
Do	5.0	58.7	42.9	18.4

*EDTA indicates ethylenediamine tetraacetic acid. Erythrocytes (5×10^6/mL) were cultured for 3 days in the presence (1:1 ratio) or absence of spleen cells, in DCCM 2 serum-free medium containing different given concentrations of EDTA. Surviving erythrocytes were counted on days 1, 2, and 3. Each value is a mean of 2 replicate values. Three-day survival of erythrocytes cultured alone is relatively poor in this experiment, as compared to other experiments, because serum-free culture medium was used in this experiment.

cytotoxicity. Results in Figure 6 show a dose-dependent lysis of erythrocytes by paraformaldehyde-fixed spleen cells in 1 or 2 days with chromium-release assay. Further evidence for involvement of a nonsecretory pathway for erythrolysis was obtained in experiments in which cell membranes prepared from spleen cells were found to lyse erythrocytes efficiently in a dose- and time-dependent manner (Figure 7). In addition, erythrocyte ghost membranes competitively inhibited the killing of erythrocytes by normal or paraformaldehyde-fixed spleen effector cells (Figure 8). These results suggest that interactions at the level of membranes were sufficient to kill erythrocytes.

3.5. Involvement of Fas/TNF Family of Molecules in EDA of Spleen Cells

Over the last decade, a family of molecules comprising Fas/Fas ligand molecules and their homologue has been shown to mediate target lysis without the involvement of soluble cytotoxic molecules [20-23]. The role of such molecules in spleen cell EDA was considered. Our repeated attempts to detect the expression of Fas and TNF receptor (TNFR) on erythrocytes by flow cytometry failed, even though faint staining with anti-Fas and anti-TNFR antibodies was occasionally obtained (results not shown). However, an indirect indication of Fas and TNFR expression on erythrocytes was obtained in experiments in which anti-Fas and anti-TNFR antibodies could opsonize erythrocytes. Results shown in Figure 9 indicate that pretreatment of erythrocytes with hamster anti-mouse Fas or TNFR antibodies (and not control hamster Ig), enhanced their depletion by spleen cells. This effect was not attributable to nonspecific membrane perturbations in anti-Fas–treated erythrocytes because pretreatment of erythrocytes with SCA (which binds erythrocytes) did not modulate their susceptibility to spleen (results not shown). The direct effect of TNF on the survival of erythrocytes was examined.

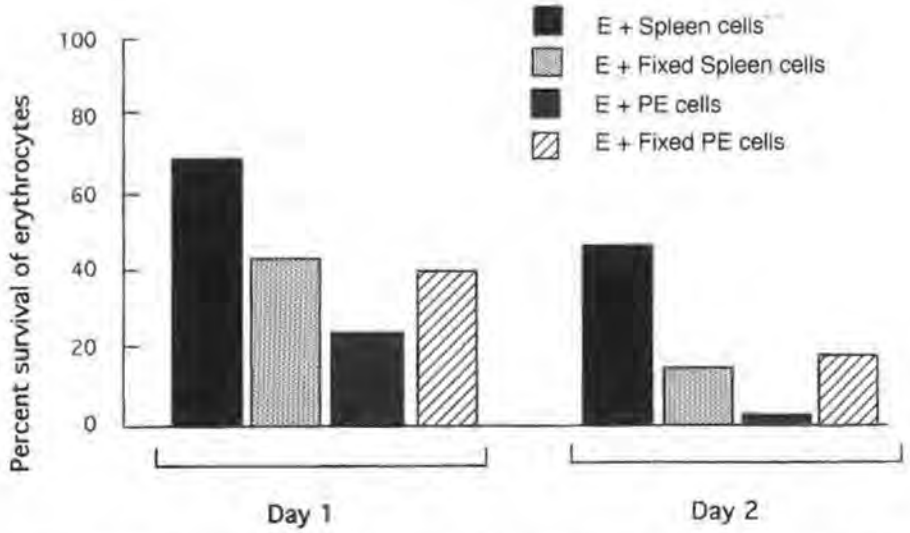

Figure 5. Killing of erythrocytes (E) by normal, paraformaldehyde-fixed spleen, and peritoneal exudate (PE) cells. Erythrocytes were incubated with erythrocyte-free spleen or PE cells, or paraformaldehyde-fixed spleen or PE cells (1:1 ratio, 5×10^6 cells/mL each) for 3 days. The number of surviving erythrocytes in culture was determined after 1 (left panel) and 2 (right panel) days of culture.

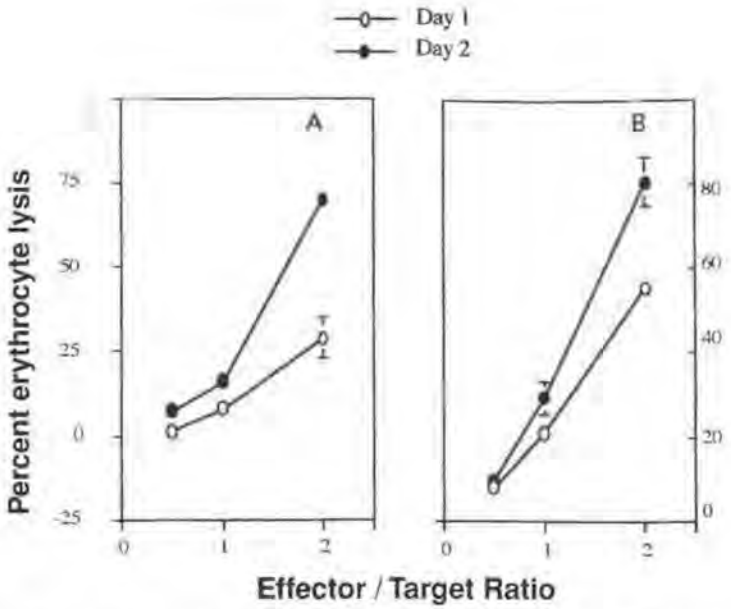

Figure 6. Lysis of erythrocytes by paraformaldehyde-fixed spleen cells in chromium release assay. Erythrocytes were labeled with sodium chromate (Cr^{51}) and incubated (10^6/assay well in 0.2 mL complete medium), with fixed spleen cells at different effector/target ratios for 1 or 2 days. Each value or erythrocyte lysis is a mean + SD of 3 replicate assay wells. Results of 2 representative experiments are shown.

Results of a representative experiment, shown in Figure 10, show that a significant dose-dependent lysis of erythrocytes was induced by TNFα. Because TNF can lyse erythrocytes directly, it is possible that EDA of spleen cells may be mediated by a membrane-expressed form of TNF. Blocking of spleen EDA by anti-TNF antibodies was attempted. Our results indicated that anti-TNF antibody did not block the EDA of unstimulated spleen cells, but could do so with SCA–induced enhancement of EDA (Figure 11).

4. Discussion

Erythrocyte numbers fell rapidly if they were co-cultured with different preparations of leukocytes. Because leukocyte preparations derived from different lymphoid organs have variable numbers of macrophages, it was likely that the phagocytosis was the cause of erythrocyte depletion in co-cultures. Accordingly, we found that PE cells having the highest concentration of macrophages had the maximum EDA. Spleen cells had moderate EDA, and thymocytes and bone marrow cells had low but significant EDA. However, plastic-adherent cells (macrophage enriched) derived from spleen cells were not enriched in EDA, whereas nonadherent spleen cells devoid of phagocytic cells had equal or more EDA than unfractionated spleen cells. Moreover, spleen cells depleted of macrophages (>90% depletion) did not show a concomitant decline in EDA. Thus, the EDA of fractionated cell preparations did not correlate well with the numbers of macrophages in them. This observation suggested that non-phagocytic mechanisms may also contribute to EDA.

Two observations indicated that T cells play a role in the EDA of leukocyte preparations: (1) very poor EDA in spleen

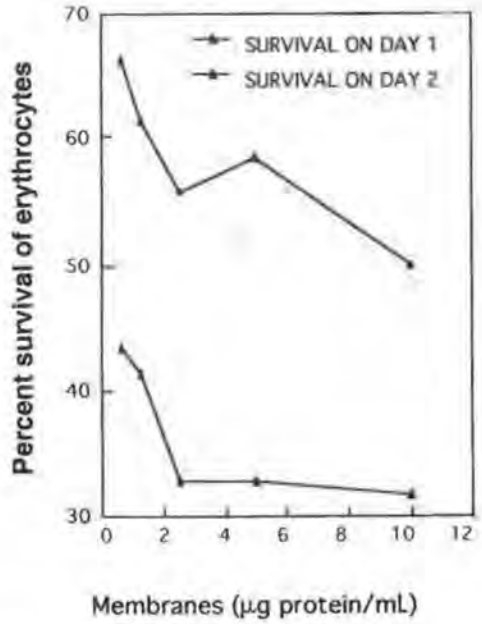

Figure 7. Dose response of the erythrocidal effect of spleen cell membrane preparations. The erythrocytes (5×10^6 cells/mL) from C57Bl6 mice were cultured with different concentrations of spleen cell–derived membrane preparations for 2 days. Percentage survival of erythrocytes was determined after 1 or 2 days of culture. Each point on the graph represents the mean of 2 replicate observations. In the absence of membranes, survival of erythrocytes was >90% on both days.

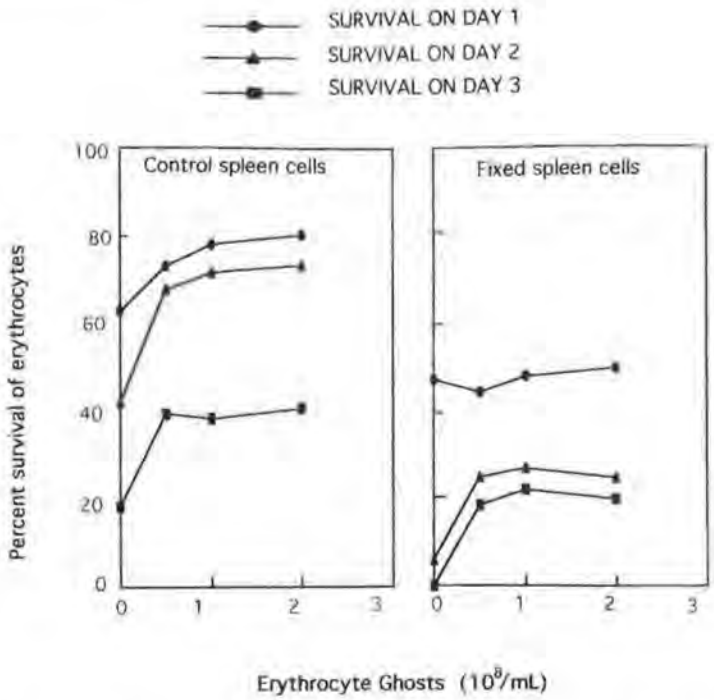

Figure 8. Inhibition of erythrocidal activity of normal and fixed spleen cells by erythrocyte ghost cells. Different numbers of erythrocyte ghosts were added to 1:1 mixtures of erythrocytes (5×10^6/mL) and normal (left panel) or fixed (right panel) spleen cells from C57Bl/6 mice for 3 days. The spleen cells were fixed by using 1% paraformaldehyde, and erythrocyte ghosts were prepared as described in Materials and Methods. The X axis denotes the equivalent of erythrocyte numbers from which ghost membranes were derived. The number of surviving erythrocytes was determined on days 1, 2, and 3 of culture. Each point on the graph depicts the mean of 2 replicate observations.

cell preparations from athymic nude mice and (2) activation of EDA by Con-A or SCA (T-cell mitogens) but not by LPS (B-cell mitogens). Direct lysis of erythrocytes by T cells may explain these observations. Some other observations, however, do not support an exclusive role of T cells in this regard. For example, enrichment of T cells by passage through NWC did not enrich EDA of spleen cells. In addition, depletion of T cells from spleen cells by anti-Thy-1 + C treatment did not deplete EDA. Although a direct erythrolytic activity of T cells may not be ruled out by these results, it is possible that T cells are required to induce EDA in other effector cell population(s) in spleen cells. NK cells also do not seem to have an exclusive role in lysing erythrocytes, because IL-2–activated spleen cells, with very high NK activity against tumor target cells [24], had poor EDA (results not shown). Our results indicate that EDA of spleen cells may not be associated with discrete subpopulations of spleen cells but may be distributed over many subpopulations.

EDA of leukocytes could either be mediated by soluble lytic factors secreted by leukocytes or by direct CMC of erythrocytes. We found no evidence for the former possibility, because culture supernatants from control or Con-A–activated spleen cells were consistently devoid of any lytic activity against erythrocytes. Several lines of evidence, however, support direct CMC of erythrocytes. Paraformaldehyde-fixed leukocyte populations, which can neither phagocytose nor release mediators, efficiently lysed erythrocytes as assessed by direct counting of residual erythrocytes or chromium-release assays of cytotoxicity. EDA associated with spleen cells increased by fixing the cells. Interestingly, fixing resulted in a reduction but not elimination of the EDA of PE cells. This result indicates that EDA of phagocytic cells such as PE cells may also have a direct CMC component. Direct CMC of erythrocytes was also supported by our results demonstrating an efficient lysis of erythrocytes by cell membranes isolated from spleen cells. This observation suggests that interaction between erythrocytes and certain membrane-expressed molecules on effector cells may be sufficient for the lysis of the former. Further evidence of such an interaction was obtained from competition experiments in which erythrocyte ghost membranes could inhibit the erythrocidal activity of normal and fixed spleen cells.

In recent years, evidence has accumulated for a nonsecretory pathway of CMC involving molecules belonging to the Fas and Fas ligand (FasL) families [20-23]. In this model, the expression of trimeric molecules like FasL and TNF on cytotoxic effector cells interacts with their corresponding ligands (Fas and TNFR, respectively) on the target cells and transmits a lytic signal [22,25]. It is also known that paraformaldehyde-fixed effector cells expressing a membrane form of the TNF family of molecules can still kill target cells expressing corresponding molecules of the TNFR family [26-28]. If this mechanism has a role in direct CMC of erythrocytes, an expression of Fas/TNFR on erythrocytes should be demonstrated. Using flow cytometry, we could not convincingly demonstrate the expression of TNFR or Fas on erythrocytes.

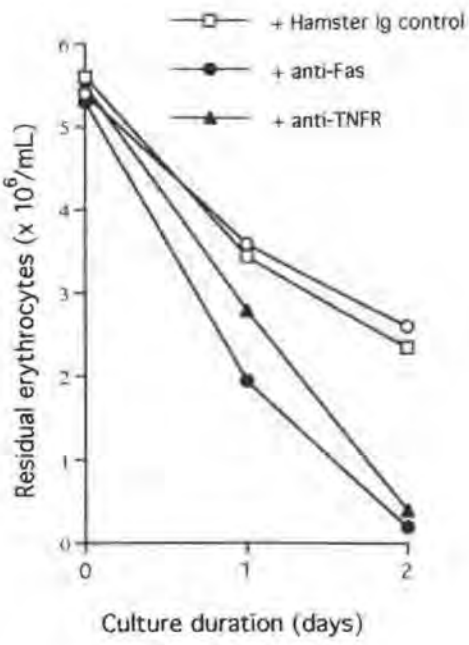

Figure 9. Lysis susceptibility of control or hamster anti-mouse Fas monoclonal antibody pretreated erythrocytes to spleen cells. The erythrocytes from C57Bl/6 mice were treated with hamster anti-mouse Fas, anti-mouse tumor necrosis factor receptor (TNFR) antibody, or control hamster immunoglobulin (all 2.5 μg/10 μL) and washed as described in Materials and Methods. Mixtures of normal erythrocytes, anti-Fas, or erythrocytes treated with anti-TNFR antibody were cultured with spleen cells from C57Bl/6 mice (1:1 ratio, 5×10^6 cells/mL each). The number of surviving erythrocytes was determined on days 1 and 2 of the culture. Each point on the graph represents the mean of 2 replicate observations.

It is possible that the expression of these molecules was too low to be detected by direct staining. It should be noted, however, that reticulocytes, the precursor nucleated cells for erythrocytes, are known to express Fas and TNFR [29,30], and it is not unlikely that some of these molecules may be retained on mature erythrocytes. Indirect evidence for such expression was provided by our experiments in which treatment with hamster anti-mouse Fas or TNFR antibodies opsonized erythrocytes for more efficient destruction by spleen cells in vitro and enhanced clearance in vivo. This effect was specific because treatment with control hamster IgG had no effect on erythrocyte destruction. Anti-Fas or anti-TNFR antibodies must be specifically bound to erythrocytes for the opsonization effect to occur, indicating some expression of the corresponding molecules on erythrocytes. Further evidence of involvement of TNF in erythrolytic activity was indicated by (1) direct lysis of erythrocytes by TNF and (2) blockage of succenyl Con-A–induced EDA in spleen cells by anti-TNF antibodies. Interestingly, TNF is known to induce anemia [31-33]; in view of our present results, direct killing of erythrocytes by TNF may be a factor in induction of anemia.

Taken together, our results provide evidence for the possibility of direct CMC of erythrocytes by leukocytes and implicate the TNF family of molecules in this effect. In nucleated target cells, there are elaborate cytoplasmic and nuclear pathways leading to cell death in response to TNF or to the Fas ligand [34,35]. Because such intercellular machinery may be lacking in erythrocytes, the mechanism of lysis of erythrocytes in response to the TNF family of molecules may be independent of the presently known interacellular pathways. Another possibility is that the role of TNF and FasL molecules may be limited to bringing together erythrocytes and effector cells. It is interesting to note that enucleated tumor targets are efficiently lysed by a nonsecretory pathway [36,37], indicating that the presence of a nucleus may not be necessary for the induction of cell death through Fas-related mechanisms.

Although there is strong evidence in the literature for phagocytosis as a mechanism of destruction of erythrocytes in vivo, alternative mechanisms of erythrocyte degradation may also operate. This may be especially true for certain pathological conditions associated with anemias. In a recent study, an enhanced in vivo clearance rate for deformed erythrocytes could not be ascribed to phagocytic activity, suggesting alternative mechanisms of erythrocyte loss in vivo [38]. In certain forms of hemolytic anemia, anti-erythrocyte auto-antibodies may be involved in the mechanism of erythrocyte loss [39]. TNF released in large amounts may cause anemia by acting at the level of erythropoiesis or also by increasing the rate of degradation of erythrocytes [40]. We have provided in vitro evidence for the possibility of CMC as a novel mechanism of erythrocyte destruction. These results may have special

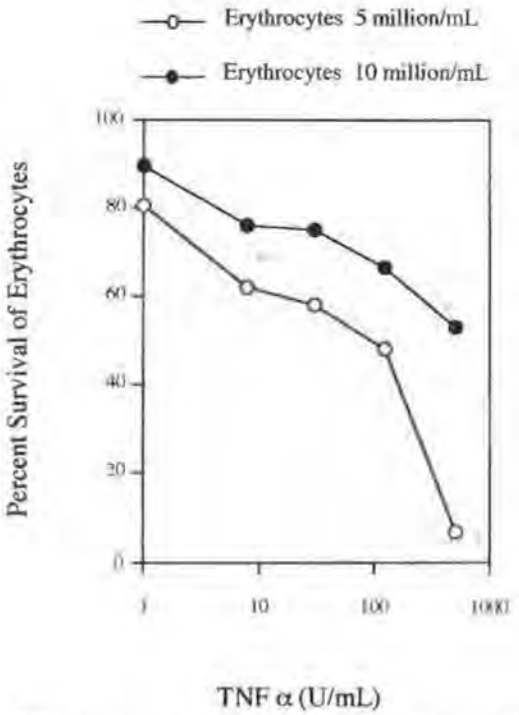

Figure 10. Dose response of murine tumor necrosis factor-α (TNFα) on the survival of erythrocytes. Erythrocytes from C57Bl/6 mice were cultured at 5 or 10×10^6 cells/mL with or without different doses of murine TNFα for 1 day, after which the number of surviving erythrocytes were counted. Each point on the graph is a mean of 2 replicate observations.

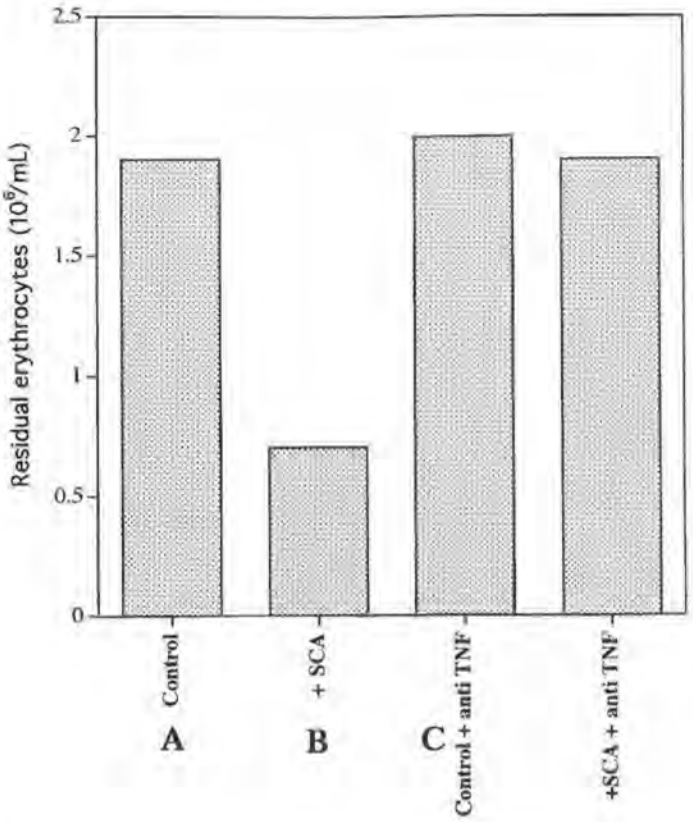

Figure 11. Blocking of succenyl con-a–induced erythrocyte depletion activities (eda) in spleen cells by anti–tumor necrosis factor-α (tnfα) antibody. Blood-derived erythrocytes and erythrocyte-free spleen mononuclear cells from C57Bl/6 mice were co-cultured (5×10^6/mL each in complete medium) for 3 days. Succenyl Con-A (SCA; 5 μg/mL) and/or anti-TNFα antibody (5 μg/mL) was added to the culture, as indicated in the table. Surviving erythrocytes at the end of the culture period were determined by hemocytometer. Each value is a mean of duplicate assay wells. Results of 3 independent experiments are shown (panels A, B, and C). SCA or anti-TNF antibody by themselves had no effect on erythrocyte survival, which was above 80% in the absence of spleen cells. Normal goat immunoglobulin used as control had no protective effect.

implications in certain types of idiopathic anemia, some of which may be attributable to augmented CMC of erythrocytes. This possibility, however, remains to be examined.

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