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DISCORDANCE AMONG CELL-MEDIATED CYTOLYTIC MECHANISMS IN CANCER PATIENTS: IMPORTANCE OF THE ASSAY SYSTEM[1]

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ADCC and SCMC directed against Chang cell targets that are mediated by lymphocytes having properties characteristic of K cells were impaired in cancer patients. In contrast, ADCC directed against CRBC targets that is mediated by both K cells and macrophages was normal in cancer patients, whereas SCMC against CRBC that is mediated primarily by macrophages was increased. Thus, there was a discordance among cytotoxic mechanisms in cancer patients with K cell-mediated cytotoxic function being impaired and macrophage-mediated cytotoxicity being enhanced. Regression analysis suggested that these perturbations of cytotoxic function occurred independently. Cancer patients had an increased proportion of circulating macrophages and a decreased proportion of eosinophils, but these abnormalities did not correlate significantly with ADCC or SCMC.

Human blood leukocytes, without prior sensitization, are cytotoxic to a variety of target cells, a phenomenon to be referred to as spontaneous cell-mediated cytotoxicity (SCMC).[3] The lytic effect of leukocytes is enhanced if the target cells are coated with specific IgG antibody, a phenomenon to be called antibody-dependent cell-mediated cytotoxicity (ADCC).

Target cells differ in their susceptibility to ADCC or SCMC by different types of effector cells. For example, chicken erythrocyte targets (CRBC) are susceptible to ADCC by granulocytes, macrophages, and a subpopulation of lymphocytes called K (killer) cells that lack surface membrane immunoglobulin (SmIg), lack or express only low-affinity receptors for sheep erythrocytes (E), and possess receptors for the Fc portion of IgG and complement (C) (1). SCMC against CRBC is mediated principally by macrophages and to a lesser extent by lymphocytes that have properties similar to K cells. (2). In contrast, Chang human liver cell targets are susceptible to both ADCC and SCMC only by K cells or K cell-like lymphocytes (3, 4).

The biologic relevance of ADCC and SCMC is unknown. In animal studies, SCMC has been associated with resistance to oncogenic viruses (5), tumor rejection (6), and homograft rejection (7). In humans, SCMC has also been postulated to be a natural cell-mediated surveillance mechanism (8), and ADCC has been shown to be operative in some patients with transitional cell carcinoma (9).

Previous reports on ADCC and SCMC effector functions in cancer patients are conflicting. ADCC has been reported to be depressed (10, 11) or unchanged (12, 13) in cancer patients, whereas SCMC has been variously reported to be either enhanced (14–16), unchanged (12), or impaired (17, 18). It is readily apparent that these discrepancies might be due to the fact that different effector cell cytotoxic functions may have been measured in these various studies since different target cells were used.

In the present study we examined both ADCC and SCMC effector function in cancer patients in simultaneous assays against two target cells known to be susceptible to lysis by different effector cells. Our results show that in many cancer patients both ADCC and SCMC mediated by K cells or K cell-like lymphocytes were impaired, whereas macrophage-mediated cytotoxicity was enhanced. This discordance among cellular cytotoxic mechanisms in cancer patients has not been reported previously and may account for some of the discrepancies that have appeared in the literature.

MATERIALS AND METHODS

Patients. One hundred patients were studied. Clinical data concerning cancer patients are summarized in Table I. Six cancer and five control patients were females. Patients receiving radiation or chemotherapy were not included in this study.

Preparation of reagents. Tissue culture medium was prepared with 10% heat-inactivated fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.) and 1% antibiotic solution containing penicillin, streptomycin, and fungizone diluted in Roswell Park Memorial Institute medium (RPMI) 1640 (Grand Island Biological Co.). This preparation will be referred to as complete medium. Normal rabbit serum (NRS) (Grand Island

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[2] Recipient of American Cancer Society Junior Clinical Faculty Fellowship No. 382.
[3] Abbreviations used in this paper: ADCC, antibody-dependent cell-mediated cytotoxicity; SCMC, spontaneous cell-mediated cytotoxicity; CRBC, chicken erythrocytes; SmIg, surface membrane immunoglobulin; E, sheep erythrocyte; NRS, normal rabbit serum; HBSS, Hanks' balanced salt solution; PBL, peripheral blood mononuclear cells; EA, erythrocyte-IgG antibody complexes; EAC, erythrocyte-IgM antibody-complement complexes.

TABLE I

Cancer patients and controls tested for spontaneous and antibody-dependent lympholysis

Patient Group	No. of Patients	Mean Age ± S.E.M.	No. of Patients Within Each Tumor Stage[a]	
			A	*B*
Control (benign urologic conditions)	59	55.5 ± 2.2		
Bladder carcinoma	13	62.7 ± 3.6	13	0
Prostate carcinoma	18	66.1 ± 1.4	11	7
Testicular carcinoma	4	34.3 ± 3.0	2	2
Renal carcinoma	3	61.3 ± 5.4	2	1
Miscellaneous[b]	3	60.3 ± 1.5	0	3
Total cancer	41		28	13

[a] Tumor stage: Stage A, localized tumor; Stage B, metastases.

[b] Two colon carcinoma; one uterine carcinoma.

Biological Co.) was heat inactivated at 56°C for 30 min and absorbed with Chang human liver cells and CRBC before use. Lyophilized rabbit anti-CRBC serum (Cappel Laboratories, Cochranville, Pa.) was heat inactivated at 56°C for 30 min before use. The antiserum was titrated in the presence of effector lymphocytes without C to determine its optimal lymphocyte-dependent cytolytic titer (3×10^{-3}). Anti-Chang cell antiserum was raised in New Zealand White rabbits by i.p. injection of 10^7 Chang cells emulsified in Freund's complete adjuvant at weekly intervals followed by bleeding 14 days after the last immunization. The anti-Chang serum was inactivated at 56°C for 30 min and titrated to determine its optimal ADCC titer (3×10^{-3}), which was used in all subsequent assays.

Preparation of target cells. Chang human liver cells (mycoplasma-free) were grown as monolayers in 25 cm^2 tissue culture flasks in a medium consisting of 10% fetal calf serum, 1% antibiotic solution, diluted in minimum essential medium (Grand Island Biological Co.). The cells were removed from the culture flask by gentle scraping with a rubber policeman, washed with Hanks' balanced salt solution (HBSS) (Grand Island Biological Co.), and labeled with 100 μCi of $^{51}Na_2CrO_4$ (sp. act. 200–500 Ci/g Cr) (New England Nuclear, Boston, Mass.) in 0.1 μl HBSS for 1 hr at 37°C in a 5% CO_2 atmosphere. Labeled Chang cells were washed twice with complete medium and resuspended to 2×10^5 cells/ml in complete medium.

Chicken erythrocytes were washed three times with HBSS and resuspended to 2×10^8 cells/ml in RPMI 1640. One-tenth milliliter of this suspension was labeled with 100 μCi of $^{51}Na_2CrO_4$ for 1 hr at 37°C in a 5% atmosphere of CO_2. Labeled CRBC were washed twice with complete medium and resuspended to 2×10^6 cells/ml in complete medium.

Preparation of effector cells. Human peripheral blood was drawn into a syringe containing 500 units of preservative-free heparin (Fellows Medical Co., Oak Park, Mich.). The blood was diluted with an equal volume of HBSS and separated on a Ficoll-Hypaque (Sigma Chemical Co., St. Louis, Mo.) density gradient as described by Böyum (19) at 200 × *G* for 40 min. Interface cells (PBL) were removed with a pipette, washed three times with RPMI 1640, and diluted to the appropriate concentrations with complete medium.

Chang cell ADCC-SCMC assay. Ten thousand ^{51}Cr-labeled Chang cells were placed into triplicate wells of Flow Microtiter plates (Flow Laboratories, Rockville, Md.) in 50-μl aliquots. To the appropriate wells, 50 μl of anti-Chang antiserum were added and the plates were incubated for 1 hr at 37°C in a 5% CO_2 atmosphere. Serial dilutions of effector PBL were added in 50 μl volumes to sensitized Chang targets to give effector-target cell ratios of 50:1, 25:1, 12:1, 6:1, and 3:1. Identical serial dilutions of effector PBL were added to Chang targets preincubated with NRS as a control (SCMC).

Additional controls included 10^4 ^{51}Cr-labeled target cells with complete medium only (spontaneous release), 100 μl 4% cetrimide (maximal release), 50 μl Chang antiserum only, and 50 μl NRS only. The plates were centrifuged at 100 × *G* for 3 min and incubated at 37°C in 5% CO_2 for 4 hr. The assay was harvested by using the Titertek Supernatant Collection System (Flow Laboratories), and the amount of ^{51}Cr released into the supernatant was determined in a Beckmann 300 system gamma counter.

The following formulas were used to calculate cytotoxicity:

$$\% \text{ Specific ADCC} = \frac{\text{Experimental release} - \text{release by highest control}}{\text{Cetrimide release} - \text{release by highest control}} \times 100$$

$$\% \text{ SCMC} = \frac{\text{Release by PBL in NRS} - \text{release by NRS alone}}{\text{Cetrimide release} - \text{release by NRS alone}} \times 100$$

Chicken erythrocyte ADCC-SCMC assay. The assay was performed similarly to the ADCC assay with Chang targets. Briefly, 50 μl aliquots containing 10^5 ^{51}Cr-labeled CRBC were placed into triplicate wells with 50 μl anti-CRBC antiserum. Peripheral blood lymphocytes were added in 50 μl volumes to give effector-target cell ratios of 5:1, 2.5:1, 1.2:1, and 0.6:1. Controls were the same as described in the Chang assay. The assay was harvested 18 hr later and percent cytotoxicity was calculated according to formulas previously given.

Preparation of effector cell fractions. Fractionated PBL (courtesy of R. P. McDermott) were separated according to a modification of the technique of Chess *et al.* (20) into T cells (E+ EA−), T cells (E+ EA+), B cells (Ig+), null cells (E− Ig−), macrophages (glass adherent), and macrophage-depleted cells (Sephadex G10 column passed) and were used as effectors to determine which cell types mediate ADCC and SCMC against Chang and CRBC targets.

Effector cell marker studies: E-rosette. Sheep erythrocytes (E) in Alsever's solution were washed three times with HBSS and resuspended to a 1% concentration. One-tenth milliliter of this suspension was added to 0.1 ml PBL (2×10^6 cells/ml) and incubated on ice for 4 hr. The pelleted cells were gently rocked and counted on a hemocytometer as E-rosette-positive if at least three SRBC adhered to a PBL.

EA-rosette. A 0.5% suspension of E were incubated at 37°C in 5% CO_2 with an equal volume of a subagglutinating titer of rabbit anti-E IgG (Cordis Laboratories, Miami, Fla.) for 1 hr. The cells were washed three times with HBSS and resuspended to a 0.5% suspension. One-tenth milliliter was added to 0.1 ml PBL (2×10^6 cells/ml) and incubated 1 hr in a 37°C water bath. Cells were counted as EA-rosette-positive if three or more EA adhered to a PBL.

EAC-rosette. A 0.5% suspension of E were incubated at 37°C in 5% CO_2 with an equal volume of a subagglutinating titer of rabbit anti-SRBC IgM (Cordis Laboratories) for 1 hr. The cells were washed three times with HBSS and resuspended to a 0.5% concentration. Equal volumes of this suspension were added to a subhemolytic titer of human serum as a source of C and incubated at 37°C in 5% CO_2 for 1 hr. The cells were washed three times with HBSS and diluted to a 0.5% suspension. One-tenth milliliter of this suspension was added to 0.1 ml PBL (2×10^6 cells/ml) and incubated 1 hr in a 37°C water bath. Cells were counted EAC-rosette-positive if three or more EAC adhered to a PBL.

Latex ingestion. Phagocytic cells were determined by adding one drop of 0.81 μ latex particles (Difco Laboratories, Detroit, Mich.) to 2×10^6 cells and incubating 45 min in a 37°C shaker bath. PBL were layered over 2 ml FCS, pelleted, and washed with HBSS. Cells were counted latex-positive if they had ingested three or more latex beads.

Immunofluorescence. Cells with surface immunoglobulin were determined by adding 0.1 ml fluorescein conjugated $F(ab')_2$ fragments of goat anti-human IgG (Cappel Laboratories) to 0.1 ml PBL (2×10^6 cells/ml) treated with latex particles as described above and placed on ice for 1 hr. The cells were washed three times with HBSS, and $SmIg^+$ cells lacking latex particles were identified by fluorescence microscopy.

Data analysis. Effector cell dose response curves were plotted depicting percent cytotoxicity as a function of effector cell concentration. Differences in cytotoxicity between patient groups were evaluated for significance by Student *t*-test. Regression analysis was used to evaluate correlations between assays.

RESULTS

Studies with effector cell fractions. Cell fractionation cytotoxicity profiles for effector cells from a normal control of ADCC and SCMC directed against Chang and CRBC targets are shown in Figures 1 and 2. Three experiments were performed on different days by using mononuclear cells from different donors. Although the absolute lytic activity varied from donor to donor, the relative lytic activities of the various enriched mononuclear cell fractions were comparable in all experiments and are in accord with previously reported studies (1, 2, 4, 21). Data shown are for one representative experiment. ADCC directed against Chang targets was mediated primarily by nonphagocytic cells that lacked SmIg, lacked high-affinity E receptors, and thus, were present in the null cell fraction; however, some lytic activity was also observed in the E+ and Fc+ fractions. ADCC against CRBC targets was mediated by both null cells and macrophages.

SCMC directed against Chang targets was mediated by nonphagocytic cells that lacked SmIg, but some of which possessed E receptors (Fig. 2). Conversely, SCMC against CRBC targets was mediated primarily by macrophages.

Effects of age on ADCC and SCMC. All available cancer patients were studied during the period of this investigation and attempts were made also to study age-matched controls concurrently. The age of both cancer and control patients was similar; however, the mean age of the cancer patients (60.9 ± 1.5) was higher than that of the control patients (55.5 ± 2.2). To determine whether this age difference affected the results of our clinical studies, a linear regression analysis was performed on the relationship between ADCC, SCMC, and age, which showed that there was no correlation between age and SCMC or ADCC with either Chang or CRBC target cells (data not shown).

ADCC studies. Sixty-nine percent of the cancer patients had ADCC against Chang cells that was less than the mean for the controls (Fig. 3*A*), and ADCC against Chang cells was significantly depressed in cancer patients as a group ($p < 0.05$) at 25:1 and at 50:1 effector-to-target cell ratios, and in terms of peak cytotoxicity (Fig. 4). However, we observed no significant difference in ADCC directed against CRBC between cancer and control patients (Fig. 5). Because our cell fractionation studies indicated ADCC against CRBC was mediated by both K cells and macrophages and because our ADCC studies with Chang targets demonstrated that K cell activity was impaired in cancer

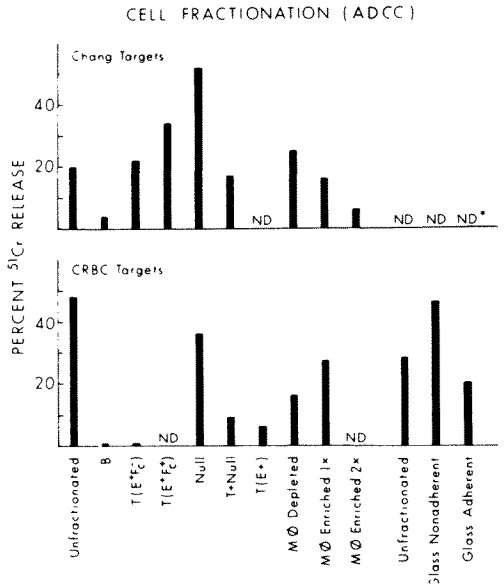

Figure 1. Antibody-dependent cell-mediated cytolysis of Chang and CRBC targets by subpopulations of peripheral blood mononuclear cells. ND, no data.

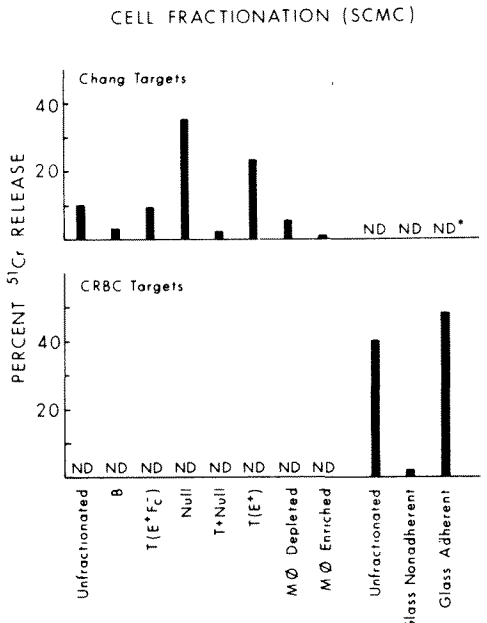

Figure 2. Spontaneous cell-mediated cytolysis of Chang and CRBC targets by subpopulations of peripheral blood mononuclear cells. ND, no data.

patients, we reasoned that macrophage-mediated cytotoxicity must be enhanced in cancer patients to account for the normal ADCC observed in cancer patients against CRBC targets.

SCMC studies. SCMC against Chang cell targets in 85% of the cancer patients was less than the mean of the controls (Fig. 3*B*) and was significantly depressed in cancer patients as a group at all effector-to-target cell ratios, as well as in terms of peak activity (Fig. 6). In contrast, SCMC against CRBC targets was greater than the mean for controls in 69% of cancer patients and was significantly enhanced ($p < 0.01$) in cancer patients as a group at all effector-to-target cell ratios (Figs. 3*B* and 7). These results provided direct evidence for a concomitant elevation of macrophage cytotoxic activity and impairment of K cell activity in cancer patients.

Leukocyte counts and effector cell marker profiles. We per-

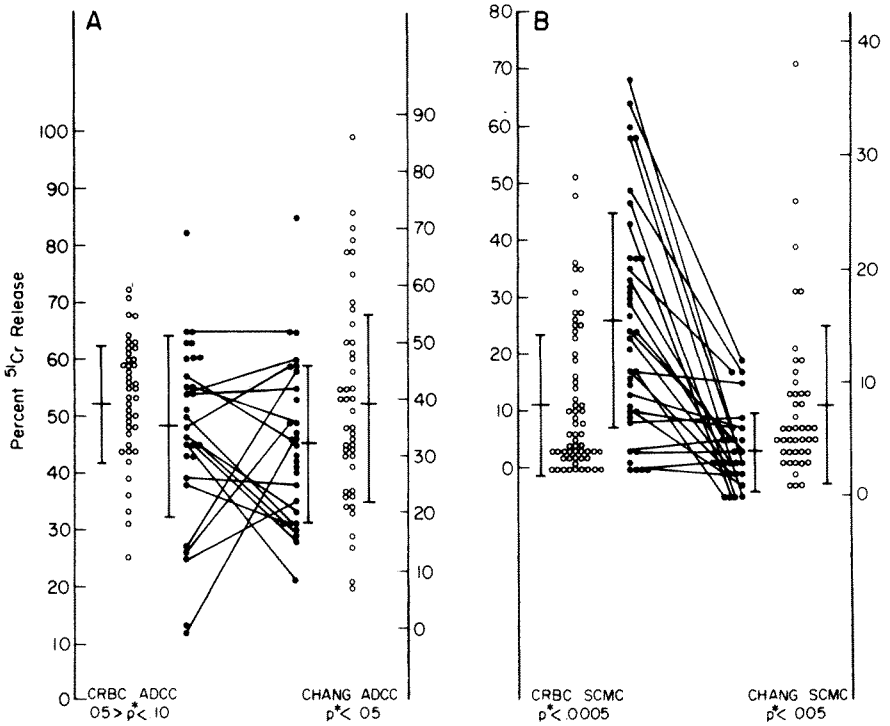

Figure 3. ADCC and SCMC against Chang cell and CRBC targets mediated by peripheral blood mononuclear cells from each cancer and control patient studied. The lines connecting assay points represent simultaneous assays. Cancer (●——●) and control (○——○) patients. *Statistical analysis by Student's *t*-test. Data show peak values.

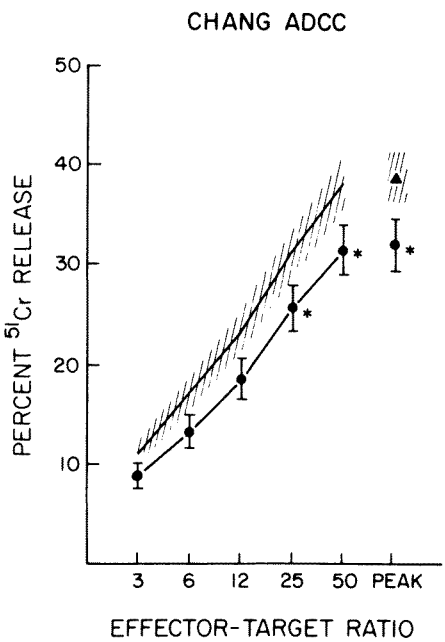

Figure 4. ADCC against Chang cell targets by peripheral blood mononuclear cells of cancer (●——●) and control (////) patients. *p < 0.05.

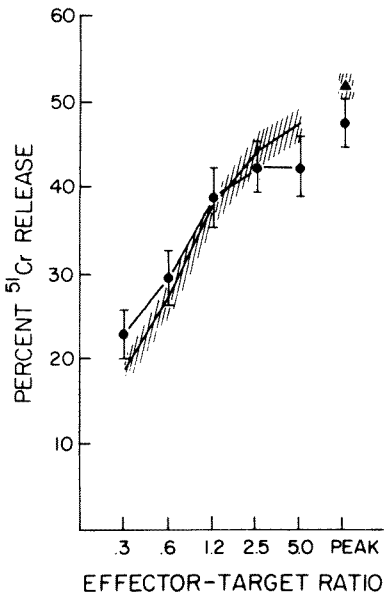

Figure 5. ADCC against CRBC targets by peripheral blood mononuclear cells of cancer (●——●) and control (////) patients.

formed complete leukocyte counts and effector cell marker profiles on all patients studied. We observed a significantly increased proportion of latex-ingesting mononuclear cells (macrophages) and a slight but significantly decreased proportion of eosinophils in cancer patients (Table II). No significant differences between cancer and control patients were noted in leukocyte counts or the proportions of cells forming E rosettes, EA rosettes, EAC rosettes, or cells with SmIg demonstrable by immunofluorescence.

DISCUSSION

Our results indicate that K cell activity as measured by both ADCC and SCMC against Chang targets was depressed relative to controls in a substantial proportion of cancer patients (Fig. 3) and was significantly depressed in cancer patients as a group (Figs. 4 and 6). Furthermore, macrophage-mediated cytotoxicity as measured by SCMC against CRBC targets was significantly enhanced in cancer patients (Fig. 7). This discordance among different cell-mediated cytotoxic functions could be demonstrated only by performing simultaneous assays with target cells that are well characterized in terms of their susceptibility to lysis by different types of effector cells.

Other possible interpretations of our data are readily apparent. First, one might argue that the impairment of both lymphocyte-mediated ADCC and SCMC was a dilutional phenomenon caused by the increased proportion of circulating macrophages observed in cancer patients. However, regression anal-

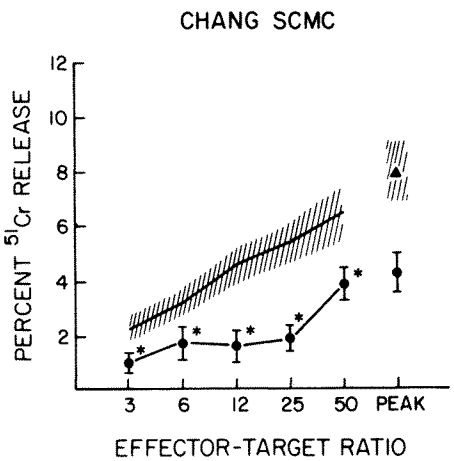

Figure 6. SCMC against Chang cell targets by peripheral blood mononuclear cells of cancer (●——●) and control (////) patients. *p < 0.05.

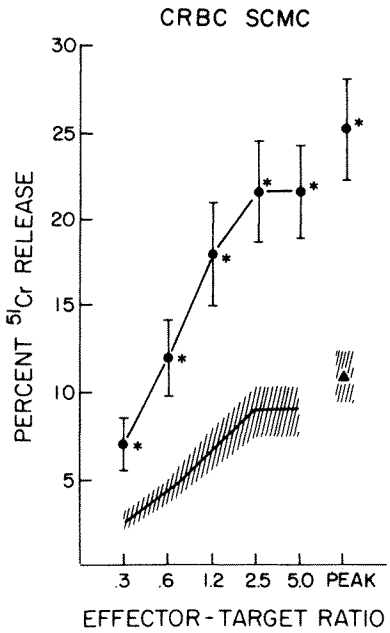

Figure 7. SCMC against CRBC targets by peripheral blood mononuclear cells of cancer (●——●) and control (////) patients. *p < 0.01.

ysis of the data revealed no significant correlation between the percentage of circulating macrophages and lymphocyte-mediated ADCC or SCMC, making it doubtful that these impairments were due only to a simple dilutional phenomenon. Second, one might argue that the perturbations of cytotoxic functions observed in cancer patients were not direct tumor-related phenomena, but rather were due to the fact that many urologic cancer patients harbor chronic urinary tract infections. This possibility seems unlikely because, although we did not study patients who were acutely ill, many of our control patients also harbored low grade genitourinary infections. Moreover, the leukocyte counts of control and cancer patients were essentially the same (Table II). Thus, the abnormalities of cytotoxic mechanisms observed appear to be a tumor-related phenomenon.

It is not known whether both ADCC and SCMC directed against a given type of target cell are mediated by the same effector cells, with the difference in lytic activity being due only to an increased lytic efficiency in the presence of antibody or whether they are mediated by separate effector cell subpopulations. Akira and Takasugi (22) presented evidence that the

TABLE II

Effector cell marker profiles and leukocyte counts in cancer patients and controls

Cell Markers	Control Patients	Cancer Patients	Significance
E rosette-positive	39.3 ± 2.5	40.7 ± 2.8	NS[a]
EA rosette-positive	7.3 ± .8	8.4 ± .7	NS
EAC rosette-positive	6.9 ± .8	6.1 ± .8	NS
Phagocytose latex	14.5 ± 1.0	18.3 ± 1.6	$p < 0.05$[b]
Surface immunoglobulin present	12.0 ± 1.1	13.4 ± 1.6	NS
Leukocyte Counts			
WBC	7300 ± 400	7600 ± 400	NS
PMN	62.8 ± 1.5	65.9 ± 1.8	NS
Lymphocytes	21.9 ± 1.4	21.7 ± 1.6	NS
Monocytes	9.8 ± 0.7	10.9 ± 1.3	NS
Eosinophils	1.9 ± 0.3	1.2 ± 0.2	$p < 0.05$[b]
Basophils	0.13 ± 0.05	0.27 ± 0.11	NS

[a] Not significant.

[b] Statistical analysis performed by Student's *t*-test.

effector cells of SCMC may be armed with antibody *in vivo* and suggested therefore that SCMC is a form of ADCC. Troye *et al.* (23) also suggested that SCMC may be a form of ADCC, but postulated that the sensitizing antibody was produced during the *in vitro* assay. In this regard, Shore *et al.* (24) were unable to increase the lytic capacity of normal effector cells by preincubating them with specific antiserum.

Evidence supporting the possibility that ADCC and SCMC are mediated by the same effector cell derives from studies showing that effectors of both ADCC and SCMC lack SmIg, lack or express only low-affinity E receptors, and possess high affinity receptors for the Fc portion of IgG (1, 3, 4, 21). Furthermore, both ADCC and SCMC can be inhibited by preincubation of effector cells with ammonium chloride (25, 26), aggregated IgG (12), and some enzymes including pronase, subtilisin, and lipase (25). However, several differences among ADCC and SCMC effector cells have been demonstrated. First, ADCC can be inhibited by Staphylococcus A protein (Staph A), whereas SCMC cannot (25). Another difference reported is that ADCC is unaffected by preincubation of effector cells with trypsin or chymotrypsin, whereas SCMC is inhibited by either enzyme (25). Finally, X-linked agammaglobulinanemia patients have been shown to have impaired ADCC but intact SCMC activity (27).

Previous reports on cell-mediated cytotoxic functions in patients with solid tumors are conflicting. Although, impairment of both ADCC and SCMC in cancer patients has been reported by some investigators (10, 11, 17, 18), others have found these cytotoxic functions to be normal (12, 13). Selective enhancement of SCMC against certain target cells also has been reported (14–16). Our results are in accord with reports in which only well defined K cell or K cell-like effector cell functions were studied (10, 18). Stratten *et al.* (10), by using macrophage-depleted effector cells against CRBC targets, observed a depression in K cell-mediated ADCC in cancer patients. Also, Pross and Baines (18) reported depressed SCMC in some cancer patients by using K 562 target cells, which are known to be lysed only by lymphocytes similar to K cells. Although other investigators observed no change in ADCC or SCMC in cancer patients, the effector cell functions operating in their assay systems were not well characterized and may have included macrophage-mediated lytic mechanisms (12, 13).

There have been reports of elevated SCMC in cancer patients

in which the effector cells were principally Fc+ null cells. In these reports, however, elevation was observed only when bladder cancer target cells were lysed by effector cells from bladder cancer patients, which suggests that in these systems tumor-specific lytic mechanisms may have been operative (14–16). Based on our results, it seems likely that the discrepancies that have appeared in the literature may be explained, at least in part, by differences in effector cell functions measured in the cytotoxicity assays used.

The control mechanisms associated with the discordance among cytotoxic mechanisms observed in this report are unknown, although several possibilities exist. The enhancement of macrophage-mediated lysis and the depression of lysis mediated by K cell-like lymphocytes could occur independently. Alternatively, K cell-like lymphocytes may be either directly or indirectly under the regulatory influence of macrophages, or both may be regulated by another type of regulatory cell. Regression analysis of our data revealed no significant correlation between macrophage cytotoxic function and K cell or K cell-like cytotoxic function. Furthermore, the association of increased macrophage-mediated cytotoxicity with decreased K cell-mediated cytotoxicity appeared to be a random event suggesting that these perturbations occur independently. Additional studies will be necessary to clarify the mechanisms involved and the biologic relevance of these phenomena.

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