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THE ROLE OF TARGET CELLS, MONOCYTES, AND Fc RECEPTOR-BEARING LYMPHOCYTES IN HUMAN SPONTANEOUS CELL-MEDIATED CYTOTOXICITY AND ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY[1]

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Human lymphocytes and monocytes were separated by velocity sedimentation at 1 × G. Approximately 60% of the lymphocytes were recovered 99.8% pure. This lymphocyte fraction (LF) did not differ significantly from the UL in the percent of Esh rosette-forming cells or the percent of cells bearing Fc- or C_3-receptors or the percent of membrane sIg-positive cells. In 16 hr CRA, the LF showed, in contrast to the corresponding UL, very weak SCMC on tumor and embryonic target cells growing in monolayers. The SCMC of the LF was completely restored by 8 to 15% autologous monocytes, which were obtained >80% pure in the same velocity sedimentation procedure. Maximal SCMC of the LF was obtained with 15 to 30% monocytes, whereas higher numbers of monocytes had suboptimal effects. SCMC of the LF could also partly be restored by monocyte culture supernatants.

The effector cell in SCMC against monolayer targets was characterized as a non-Esh rosette-forming, membrane sIg-negative, Fc-receptor-bearing lymphocyte. The SCMC of the LF depleted of Fc-receptor-bearing lymphocytes could not be restored by autologous monocytes, indicating that the Fc-receptor-bearing lymphocyte is the actual effector in SCMC, but that monocyte help mediated by soluble factors is required.

In contrast, K562 and MOLT-4 cells that grow in suspension cultures, and antibody-coated Chang cells were lysed by LF cells in the absence of monocytes. In spite of the reactivity of the LF in ADCC, no clear evidence was found that antibodies played a role in SCMC or that monocyte help was mediated by the transfer of cytophilic antibodies. Preincubation of the effectors with $F(ab')_2$ fragments of goat anti-human Fab antibodies or the presence of this reagent in the test did not specifically inhibit SCMC targets growing in monolayer and suspension cultures. However, ADCC in which HLA-A_2-positive target cells and anti HLA-A_2 antiserum were used was completely blocked by our anti-human IgG reagent.

These results indicate that SCMC is probably not a form of ADCC and that SCMC and ADCC on cells growing in monolayers are mediated by different mechanisms, the first requiring monocyte help and the latter not. In addition, the monocyte-independent SCMC of the LF on K562 and MOLT-4 suggests that the SCMC mechanism may vary with the type of target cell used in the assay (targets growing in monolayers *vs* targets from leukemic origin growing in suspensions). However, the possibility that different types of effector cells are involved in both systems may not be excluded.

Lymphocytes from healthy individuals can spontaneously lyse a large variety of target cells derived from tumor, normal, and embryonic tissue *in vitro* (1, 2). The effector cells in this spontaneously cell-mediated cytotoxicity (SCMC)[4] have been shown to be similar in many respects to the killer cells in antibody-dependent cellular cytotoxicity (ADCC), since both types of effector cells lack surface membrane immunoglobulin (sIg), lack or express only low-affinity receptors for sheep erythrocytes (Esh), but possess receptors for the Fc portion of IgG (3–9). Furthermore, both ADCC and SCMC can be inhibited by preincubating the effectors with aggregated IgG (4, 8) and some enzymes like pronase, subtilisin, and lipase (9). In addition, it has been reported that antibodies may play a role in SCMC. Therefore, it has been suggested that SCMC is actually a form of ADCC (10–13); however, Pape *et al.* (13) report also that antibody-independent SCMC occurs simultaneously. In contrast, Kay *et al.* (14) found no clear evidence in support of a role of antibody in SCMC. In addition, several differences between SCMC and ADCC effectors have been described. SCMC but not ADCC is inhibited by treating the effector cells with trypsin or chymotrypsin (9). ADCC can be inhibited by staphylococcus A protein, whereas SCMC cannot (9). SCMC is significantly inhibited by dexamethasone, but

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[4] Abbreviations used in this paper: LF, lymphocyte fraction; UL, unfractionated lymphocytes; MF, monocyte fraction; CRA, ^{51}Cr release assay; MCA, microcytotoxicity assay; SCMC, spontaneous cell-mediated cytotoxicity; ADCC, antibody dependent cellular cytotoxicity; MCS, monocyte culture supernatant; 2ME, 2-mercaptoethanol; Esh, sheep erythrocytes; EoxA, ox erythrocytes sensitized with anti Eox IgG; EshAC, sheep erythrocytes sensitized with anti Esh IgM and mouse complement; sIg, surface immunoglobulin; DMEM, Dulbecco's minimal essential medium; WGA, wheat germ agglutinin.

ADCC is not (15). Finally, SCMC is not affected in X-linked a-γ-globulinemia patients, whereas ADCC activity was impaired (16). In the present paper, we demonstrate that a majority of the effector lymphocytes in SCMC require monocyte help, which is not needed for ADCC, adding further evidence that different mechanisms are involved in SCMC and ADCC. Furthermore, we present evidence that the SCMC mechanism may vary with the type of target cell used in the assays.

MATERIALS AND METHODS

Media and reagents. All cell cultures and tests were carried out in Dulbecco's modification of Eagle's minimal essential medium (DMEM), supplemented with 3.8 g $NaHCO_3$/1 liter, penicillin (100 IU/ml), streptomycin (100 μg/ml), and fungizone (2.5 μg/ml), and 10% heat-inactivated (30 min, 56°C) fetal calf serum (FCS), all purchased from Gibco-Biocult, Glasgow, Scotland. This medium is designated as culture medium. Carrageenan (type V, iota carrageenan), concanavalin A (Con A), and wheat germ agglutinin (WGA) prepared from *Triticum vulgaris* were obtained from Sigma Chemical Co., St. Louis, Mo. Phytohemagglutinin (PHA) was purchased from Wellcome, Beckenham, Kent, England, and 2 mercaptoethanol (2ME) from Eastman Kodak, Rochester, N. Y. Fluoresceinated goat anti-human Ig came from Cappell Laboratories, Downington, Pa.

Target cells. The T-24 bladder carcinoma cell line (17), two melanoma cell lines established in our laboratory (NKI-1 and NKI-10), two neuroblastoma cell lines (Gil and Sto) established and kindly provided by Dr. Lindsay Ogg, University Dept. of Pathology, Glasgow, Scotland, and EK, an embryonic kidney cell line obtained from Dr. Dekking (Laboratory of Gezondheidsleer, University of Amsterdam) were used as target cell sources for SCMC assays. Chang liver cells and NKI-10 cells were used as target cells in ADCC. In the latter situation, anti-HLA-A_2 antiserum (kindly provided by G. de Lange, Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam) was used as antibody source, since the NKI-10 cells expressed HLA-A_2 determinants. All these cells were growing in monolayer cultures. The K562 cell line originally derived from blast cells in a pleural effusion of a patient with chronic myeloid leukemia (18) and MOLT-4, a T cell leukemia cell line (19) were maintained as suspension cultures. The K562 cells were cultured in the absence of antibiotics, and both K562 and MOLT-4 were subcultured 1 day before testing in SCMC.

Effector cells. Mononuclear leukocytes were isolated from defibrinated blood of healthy donors by centrifugation on a Ficoll-Hypaque solution (12 min at 1500 × G). These cells were further separated according to size by velocity sedimentation at unit gravity with a modified version of the LACS-1 cell separator (De Koningh, Arnhem, The Netherlands) as described previously (20). Sixty percent of lymphocytes applied to the gradient were recovered in a fraction that had a sedimentation velocity of 20 to 36 mm/hr (20). This fraction contained 99.8% pure lymphocytes and was therefore designated as lymphocyte fraction (LF). Fractions 2, 3, and 4 had sedimentation velocities of 36 to 40, 40 to 44, and 44 to 48 mm/hr and contained varying mixtures of lymphocytes and monocytes. These fractions contained on a per effector cell basis approximately 30% of the SCMC of the unfractionated lymphocytes. About 40% of all monocytes were recovered in the monocyte-enriched fraction (MF), which had a velocity sedimentation of 48 to 62 mm/hr and contained 83 ± 4% monocytes. After fractionation the cells were washed three times and resuspended in the appropriate concentration in culture medium.

Characterization of LF and MF. The percentage of monocytes present in the LF and MF was estimated in cytocentrifuge preparations stained for nonspecific esterase (21) and with Wright's stain. The monocytes in the MF were further characterized by phagocytosis of fluorescent carboxylated beads (1.6 μ diameter, Polysciences, Warrington, Pa.) and by electronic sizing with a Coulter counter, model ZF, supplemented with a pulse height analyzer (Chanelyzer, model C 1000) (22).

Characterization of subpopulations of lymphocytes in the different fractions. Characterizations of lymphocyte subpopulations were carried out in conjunction with the large-scale preparative rosette procedures used to fractionate lymphocyte subpopulations, since it was shown previously that the percentages of rosettes thereby obtained did not differ from the percentages of rosettes observed in standard micro procedures (23).

Esh-rosettes. Esh-rosettes were prepared and counted as described previously (23). Briefly, 10^7 lymphocytes and 5×10^8 Esh in 10 ml volumes were centrifuged (5 min at 150 × G) and incubated for 1 hr at room temperature. Eight milliliters of the supernatant were discarded, and the cell sediment was carefully resuspended in the remaining 2 ml of supernatant.

EoxA rosettes. EoxA rosette-forming cells were characterized by rosette formation with ox erythrocytes (Eox) coated with a maximal subagglutinating titer of rabbit anti-EoxIgG (20).

EshAC rosettes. EshAC rosettes were prepared by coating Esh with a maximal subagglutinating titer of rabbit anti-Esh IgM, which was free of IgG as was judged by immunoelectrophoresis, and fresh BALB/c mouse serum as complement source. The EshAC rosettes were resuspended by vortexing.

Lymphocytes with sIg. Surface immunoglobulin-bearing cells were detected by membrane fluorescence as described previously (20). For the depletion of these cells, a rosette-formation procedure employing Esh coated with rabbit anti-human Ig was used. The rabbit anti-human IgG was coupled to the Esh by incubating equal quantities of 0.1% rabbit anti-human IgG, 0.1% $CrCl_3$ and packed Esh for 6 min at room temperature. The IgG-coated Esh were mixed with lymphocytes (20:1) spun for 5 min at 150 × G and resuspended vigorously to break up the less firmly bound EoxA rosettes. The remaining rosettes were designated as sIg rosettes.

Separation of rosette-forming cells from nonrosette-forming cells. The EoxA and EshAC rosetting procedures were also carried in 10 ml volumes containing 10^7 lymphocytes and 5×10^8 EoxA or EshAC in 50 ml round-bottomed tubes. After the incubation period, the Eox and EshAC rosettes were resuspended as described above for the Esh rosettes. The EshAC rosettes were more vigorously resuspended. These cell suspensions were diluted to 15 ml with culture medium, underlaid with 10 ml of Ficoll-Hypaque, and centrifuged (20 min at 400 × G at the interface). The effectiveness of the depletion and enrichment of the different lymphocyte subpopulations was assessed as described previously (23). The purity of the various subpopulations is briefly presented in the *Results* section. Since our methods did not change, we can assume that the same degree of purity was obtained in the present rosette separation procedure.

Carrageenan treatment. Mononuclear cell suspensions were incubated at 37°C for 3 to 4 hr with carrageenan (300 μg/ml) and 5% CO_2 and agitated at regular intervals. After the incubation period, the cells were washed three times and resuspended in culture medium.

Monocyte culture supernatants (MCS). Monocyte culture supernatants were obtained from cultured MF cells. 4×10^5 MF cells in 0.2 ml culture medium were seeded per well of microtiter

plate (Falcon Plastics, No. 3040, Oxnard, Calif.). After overnight incubation (37°C, 5% CO_2) the wells were vigorously washed to remove nonadherent cells (in some experiments, this washing was carried out 3 hr after the MF cells had been seeded). Fresh culture medium (0.25 ml) was added, and the plates were incubated for another 72 hr (37°C, 5% CO_2). The supernatants of these confluent monolayers were collected, centrifuged 10 min at 2000 × G, and stored at −80°C until testing.

Anti-human IgG antiserum. The anti-human IgG antiserum preparation was obtained from Dr. Snoijink (Nordic, Immunological Laboratories, Tilburg, The Netherlands) and contained $F(ab')_2$ fragments of purified goat IgG antibodies to Fab fragments of pooled human IgG. This reagent (referred to as anti-human IgG antibody) contained 11 mg of purified antibody protein/ml, and the antibody activity was directed against Fab of both light chain types. The antiserum was prepared by immunizing a goat with purified Fab fragments obtained by papain degradation of highly purified human IgG. The IgG fraction of the antiserum was separated by sodium sulphate precipitation and purified by passage over DEAE-Sephadex A50. The anti-Fab antibodies were isolated from this IgG fraction by affinity chromatography with insolubilized Fab of polyclonal human IgG used as immunoadsorbent.

The $F(ab')_2$ fragments of these purified antibodies were obtained after pepsine digestion and Sephadex G150 passage. This preparation contained no detectable Fc fragments or undigested IgG as judged by immunoelectrophoresis and double immunodiffusion. Anti-Fab activity was demonstrated by immunoelectrophoresis against a mixture of Fab and Fc fragments of human IgG. No precipitation activity against the Fc fragments could be detected. The antibody activity was further tested by direct cytoplasmic immunofluorescence of monoclonal human bone marrow plasmocytes after a part of the reagent had been conjugated with fluorescein and rhodamine. The conjugated preparation clearly recognized lymphoid cells containing IgG, IgM, IgA, or IgD of either L chain type. Furthermore, the absence of antibody activity to non-Ig serum proteins could be established in immunoelectrophoresis against pooled normal human serum. In addition, this reagent did not inhibit the sensitization of Chang cells with rabbit anti-Chang IgG. As a control serum, immunoadsorbent-purified $(F(ab')_2$ fragments of goat anti-rabbit IgG (purchased from Nordic) were used. In this preparation, containing 10.2 mg protein/ml, no Fc fragments or undigested IgG could be detected. No cross-reactivity in Ouchterlony gel diffusion tests was observed between the anti-human IgG reagent and the goat anti-rabbit IgG.

^{51}Cr release assays (CRA) for SCMC and ADCC. The CRA from SCMC and ADCC were carried out simultaneously by using the same effector cell fractions. Confluent monolayers of target cells were trypsinized, and 10^4 target cells in 0.2 ml culture medium were seeded per well of microtiter test plates (Falcon Plastics, No. 3040) and incubated overnight at 37°C and 5% CO_2. The cells were labeled with 4 μCi ^{51}Cr (sodium-chromate, New England Nuclear, Boston, Mass.) per well and incubated for an additional 4 hr. After this incubation period, the cells were washed three times with approximately 0.25 ml culture medium and used as targets in cytotoxicity tests.

For ADCC assays, rabbit anti-Chang liver cell antibody or anti-HLA-A_2 antibody was added during the last 20 min of the incubation period with ^{51}Cr. Free ^{51}Cr and free antibody were removed by three washings with culture medium. In the ADCC assays, Chang liver cells and NKI-10 cells cultured in the absence of antibody were used as controls to measure the SCMC on these cells. In the SCMC assays on target cells growing in monolayers and the ADCC on NKI-10 cells, 4×10^5 effector cells were added per well. In the ADCC assay on Chang cells, 1 or 2×10^5 effectors were added per well. All tests were carried out in triplicate. After 16 hr incubation at 37°C and 5% CO_2, 100 μl of the supernatant were harvested and counted in a type 5260 gamma counter (Packard Instrument Company, Downers Grove, Ill.).

Target cells growing in suspension cultures were labeled by incubating 1×10^6 cells in total volume of 0.4 ml culture medium with 100 μCi ^{51}Cr for 45 min at 37°C. The labeled cells were washed three times with 30 ml of culture medium and resuspended at 1×10^5 cells/ml. The SCMC assays were carried out in U-bottomed microtiter plates (Linbro Scientific, No. 78-013-05, Hamden, Conn.) that contained 10^4 labeled target cells (in 100 μl) and 2×10^5 effectors (in 100 μl) per well (effector-target cell ratio 20:1). Each test was set up in triplicate. The plates were centrifuged for 2 min at 200 × G and incubated at 37°C for 4 hr with 5% CO_2. After this incubation period, 100 μl supernatant were collected and counted as described above. Cytotoxicity in all assays was expressed as the percentage of specific ^{51}Cr release, which was calculated as follows: $A - B/T - B \times 100$, where A = mean cpm of the test sample, B = mean cpm of the spontaneous ^{51}Cr release (i.e., ^{51}Cr released by the labeled target cells in the medium in the absence of effector cells), and T = mean cpm of the maximal ^{51}Cr release that was obtained after addition of 1% Triton-X to the labeled target cells. The labeling of the target cells growing in monolayers resulted in 1 to 3 cpm per cell. The mean spontaneous release of the various target cells over a 16-hr period of incubation was T-24: 18 ± 3%, NKI-1: 15 ± 3%, Chang: 16 ± 3%, NKI-10: 13 ± 3%, EK: 20 ± 4%; and during a 4-hr incubation period for K562: 8 ± 2% and MOLT-4: 10 ± 2%.

RESULTS

SCMC of unfractionated leukocytes (UL) and LF and MF cells. The SCMC of the UL and the LF and MF cells from 14 healthy donors is shown in Figure 1. LF cells, which were highly depleted of monocytes (average monocyte contamination 0.2%), showed insignificant or very weak cytotoxic effects in contrast with the UL. Weak SCMC was also scored with the MF, which contained over 80% monocytes. Most of the contaminating cells in the MF were lymphocytes (Table I). The strongest SCMC was measured on EK and T-24 cells, whereas the NKI-10 cells were rather resistant for lysis. Since characterization of the UL and LF revealed no significant differences in the percentages of Esh, EoxA, and EshAC rosette-forming and sIg-bearing lym-

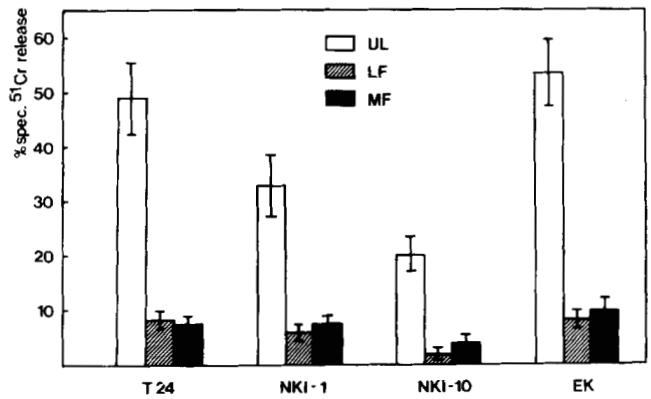

Figure 1. SCMC of the unfractionated lymphocytes (UL) and the corresponding lymphocyte fractions (LF) and monocyte fractions (mean ± S.E.M. of 14 healthy donors).

TABLE I

Characterization of the unfractionated lymphocytes (UL) and the five different fractions obtained after velocity sedimentation at 1 × G (mean ± S.D. of nine experiments)

	UL	LF	Fr2	Fr3	Fr4	MF
Sedimentation velocity, mm/hr[a]		20–36	36–40	40–44	44–48	48–62
% Monocytes[b] (esterase)	23 ± 4	0.2	2.8–0.4	14 ± 4	50 ± 5	83 ± 4
% Monocytes[c] (phagocytosis)	20 ± 5	0.1	0.6	11 ± 2	38 ± 2	74 ± 5
% Monocytes[d] (electronic sizing)	22 ± 4			18 ± 4	54 ± 5	87 ± 4
% Lymphocytes	77 ± 4	99.8	97.2	86 ± 4	50 ± 5	16 ± 4
% Lymphocytes forming E rosettes	51 ± 6	57 ± 5	47 ± 4	36 ± 4	17 ± 4	9 ± 3
% Lymphocytes with Fc receptors	16 ± 3	17 ± 4	24 ± 3	27 ± 4	19 ± 4	10 ± 3
% Lymphocytes with C_3 receptors	18 ± 4	20 ± 3	23 ± 3	23 ± 4	12 ± 3	5 ± 2
% Lymphocytes with sIg	6 ± 2	7 ± 2	9 ± 2	9 ± 2	5 ± 2	3 ± 1
% Granulocytes	0.5	0	0	0	0	1

[a] Calculated as described in Reference 20.
[b] The results are expressed in percentages of all cells in a given fraction.
[c] Phagocytosis is measured in seven experiments.
[d] Electronic sizing is measured in three experiments.

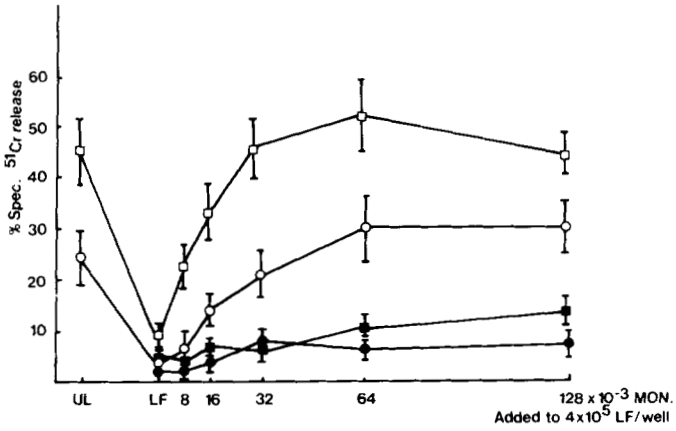

Figure 2. Restoration of SCMC of LF cells by autologous monocytes (mean ± S.E.M. of four experiments). □—□, SCMC on T-24; ■—■, SCMC on T-24 after addition of carrageenan-treated monocytes; ○—○, SCMC on NKI-1; ●—●, SCMC on NKI-1 after addition of carrageenan-treated monocytes.

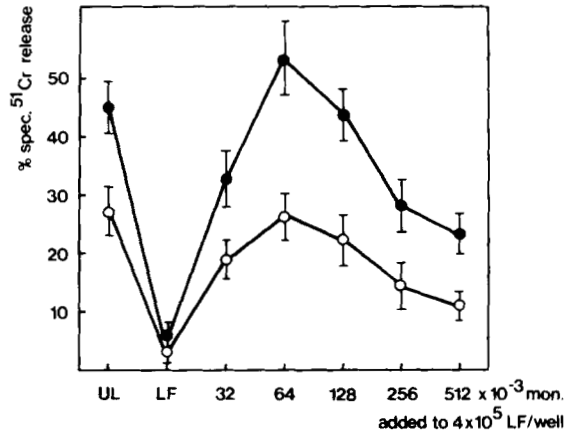

Figure 3. The effect of relatively high numbers of autologous monocytes on the restoration of SCMC of LF cells. ○—○, SCMC on T-24; ●—●, SCMC on NKI-1.

phocytes (Table I), these results suggested that the loss of SCMC of the LF was due to the depletion of monocytes.

Restoration of SCMC of the LF with autologous MF cells. To determine whether the loss of SCMC with the LF cells actually was due to depletion of monocytes, reconstitution experiments were carried out by adding back graded numbers of autologous MF cells (Fig. 2). Addition of 4% monocytes to the LF resulted in a significant restoration of the SCMC on T-24 and NKI-1 targets, whereas approximately 8 to 15% monocytes were sufficient to restore the SCMC of the LF to the level achieved with the corresponding UL. The maximal restoration of SCMC, which was higher than obtained with UL, was observed after addition of 15 to 30% monocytes to the LF. Higher concentrations of monocytes had suboptimal effects on the restoration of SCMC (Fig. 3). MF cells in the absence of LF cells were not cytotoxic.

Since the MF used as monocyte source for our reconstitution experiments contained approximately 20% lymphocytes, the possibility must be excluded that the restoration was mediated not by the monocytes in these MF but by the noncytotoxic lymphocytes present. To evaluate this possibility, the MF cells were preincubated with carrageenan, a polygalactan that selectively destroys monocytes/macrophages (24). Carrageenan-treated monocytes were ineffective in the restoration of SCMC on the LF on T-24 and NKI-1 (only very low levels of SCMC were observed after addition of 30% carrageenan-treated monocytes), indicating that the helper effects were mediated by monocytes and not by the contaminating lymphocytes in the MF (Fig. 2, and see Fig. 6). LF cells preincubated with carrageenan showed after addition of autologous monocytes good responses to mitogens, indicating that the lymphocytes were not affected by the carrageenan treatment (de Vries *et al.*, unpublished findings). Intact but formalin-killed monocytes were also ineffective in restoring the SCMC to the LF (see Fig. 6).

Restoration of SCMC of the LF by allogeneic MCS. Since we demonstrated that SCMC of the LF required the presence of viable monocytes, we next tried to determine whether the restoration of SCMC of the LF required monocyte-lymphocyte interactions or whether it could be mediated by soluble factors produced by these monocytes. Experiments were carried out in which allogeneic MCS were added to the LF. Supernatants from confluent monocyte monolayers diluted 1:2 or 1:1 in fresh culture medium showed a significant restoration of SCMC of the LF (Fig. 4). The restorative effect of the MCS were, how-

ever, always weaker than the effects obtained with addition of viable monocytes, and never exceeded 60% of the SCMC achieved with the corresponding UL. Monocytes or MCS could not be replaced by 2ME, a substance that can replace monocytes/macrophages as helper cells in the antibody production by lymphocytes *in vitro* (25, 26). 2ME also had no supplementary effect on the restorative effects of MCS.

Identification of the effector cell in SCMC on target cells growing in monolayers. The effectiveness of our procedures used for depletion and enrichment of the different lymphocyte subpopulations was shown in detail previously (23). Rosette formation and fractionation procedures were carried out on UL partially depleted of adherent cells by adherence to plastic Petri dishes. Staining for nonspecific esterase demonstrated that these populations still contained an average of 6% monocytes. SCMC was not significantly influenced by this incomplete removal of adherent cells (Fig. 5). Depletion of Esh rosette-forming cells resulted in slightly increased SCMC. The Esh rosette-depleted population contained an average of 4% Esh rosette-forming cells, whereas the Esh rosette-enriched populations contained over 90% Esh rosette-forming cells. Lymphocytes in these Esh rosette-enriched populations were recovered after lysis of the Esh, and they showed no or very weak cytotoxic effects. The lack of SCMC of the Esh rosette-forming lymphocytes could not be attributed to the NH_4Cl/Tris buffer that was used to lyse the Esh (8), because Esh rosette-forming cells freed of Esh by vortexing at 37°C and 1 × G sedimentation in the LACS-I apparatus were equally nonreactive (results not shown). SCMC was greatly reduced by the depletion of Fc receptor-bearing cells (EoxA rosettes). The EoxA rosette-depleted cell population was contaminated with an average of 3%

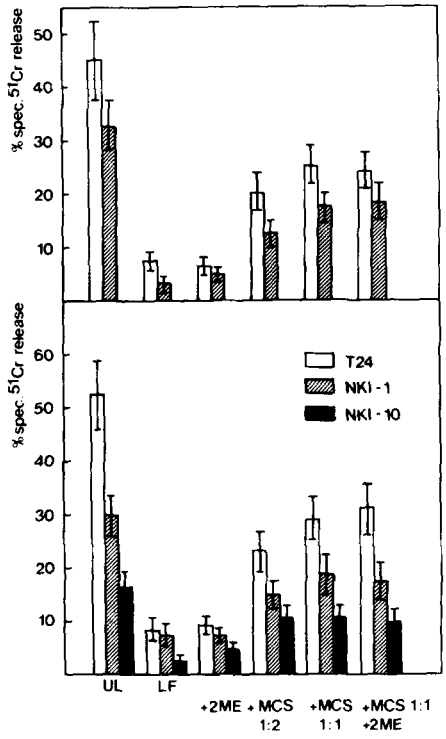

Figure 4. Restoration of SCMC of LF cells by two different allogeneic supernatants, MCS-1 and MCS-2. *Upper*, MCS-1 added to LF cells from three different donors; *lower*, MCS-2 added to LF cells from three different donors. MCS 1:2, 1 part of MCS diluted in 2 parts of culture medium; MCS 1:1, MCS diluted 1:1 in culture medium. MCS-1 and MCS-2 had no cytotoxic effects on T-24 and NKI-1 targets in the absence of LF cells.

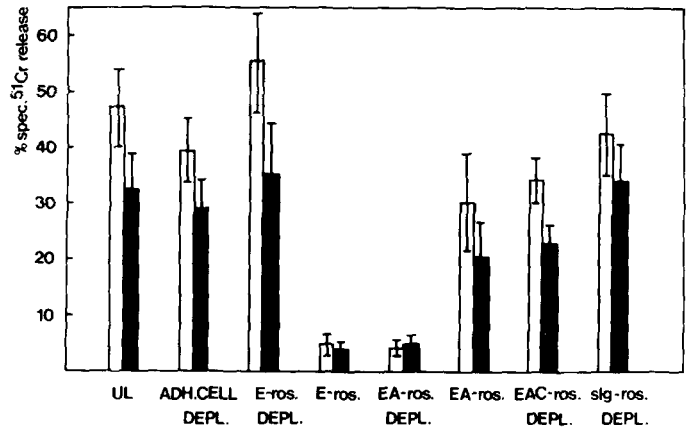

Figure 5. Identification of the effector cell in SCMC. □, SCMC on T-24 target cells (mean ± S.E.M. of four different donors); ■, SCMC on NKI-1 target cells (mean ± S.E.M. of three different donors).

Fc receptor-bearing cells and contained 80% Esh rosette-forming cells (23). After lysis of the erythrocytes, the Fc receptor-enriched fraction showed SCMC that was, however, weaker than the SCMC of the UL. The reason for this is unclear but may be attributed to Eox ghost fragments still present on the membranes of the lymphocytes. Depletion of C_3 receptor-bearing cells (EshAC rosettes) was very efficient (contamination with EshAC rosette-forming cells was 3%) and resulted in a partial reduction of SCMC. Removal of sIg-bearing lymphocytes by sIg rosettes (leaving 1% sIg-positive cells as judged by immunofluorescence) did not influence SCMC. It was shown previously that monocytes were recovered in all rosette-depleted as well as in the rosette-enriched fractions. The monocyte contaminations varied between 2 and 6% (23). These results show that the effector cell in SCMC can be characterized as a non-Esh rosette-forming, sIg-negative Fc-receptor bearing lymphocyte, a proportion of which probably also bear C_3 receptors.

Addition of monocytes to LF cells depleted of Fc receptor-bearing cells. In spite of the presence of Fc receptor-bearing lymphocytes in the LF, this fraction did not show SCMC. Since 60% of all lymphocytes were recovered in the LF, it seems highly unlikely that the Fc receptor-bearing cells in the LF differ from the Fc receptor-bearing cells in the UL that were the effectors of SCMC. However, to exclude this possibility, monocytes were added to LF cells that were depleted of Fc receptor-bearing lymphocytes. In this situation, monocytes were far less effective in the restoration of SCMC (Fig. 6). Only an insignificant restoration of SCMC was observed after addition of the highest monocyte concentration (about 30%). These results indicate that the Fc receptor-bearing cells are the actual effectors in SCMC when monocytes are added back to the LF.

SCMC and ADCC profiles of the five different effector cell fractions obtained by 1 × G sedimentation. The five different fractions (LF, fraction 2, fraction 3, fraction 4 and MF) obtained after 1 × G sedimentation, and the UL from which they were obtained, were tested in parallel for SCMC on T-24, NKI-1, NKI-10, MOLT-4, and K562 cells and for ADCC on Chang liver cells (Fig. 7). The different lymphoid subpopulations in the fractions 2, 3, and 4 are characterized in Table I. SCMC or eight different donors was measured in parallel on T-24, NKI-1, and NKI-10, whereas five of these donors were also tested on K562 and for ADCC on Chang liver cells. Four donors were tested on MOLT-4 cells. Compared with the UL, the LF cells (containing 0.1% monocytes) showed no or very weak SCMC on T-24, NKI-

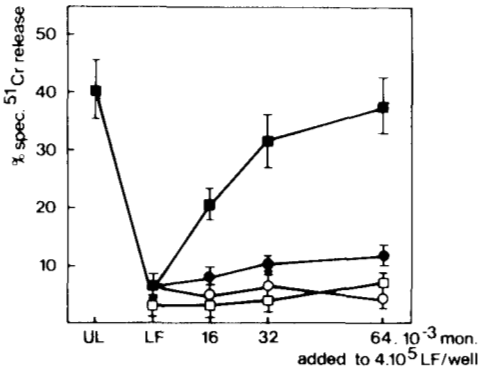

Figure 6. Addition of autologous monocytes to LF cells depleted of Fc receptor-bearing cells. SCMC is measured on T-24 target cells. ■——■, viable monocytes added to LF; ●——●, monocytes pretreated with carrageenan added to LF; ○——○, intact, but formalin-killed monocytes added to LF; □——□, viable monocytes added to LF depleted of Fc receptor-bearing lymphocytes.

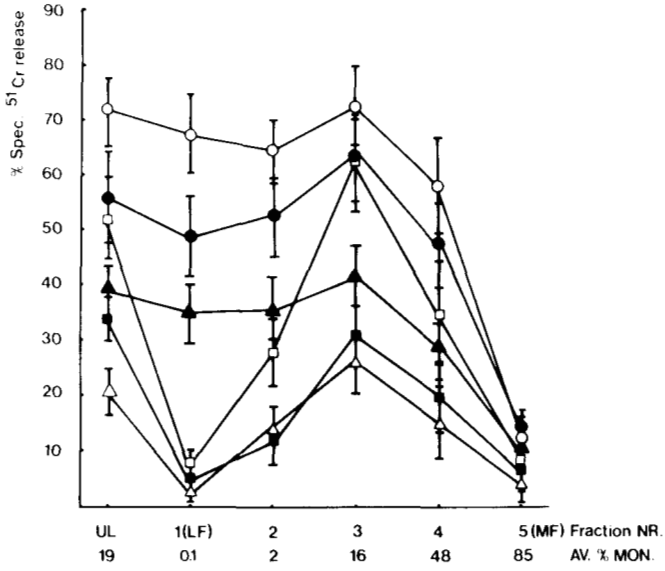

Figure 7. SCMC and ADCC profiles of the five different mononuclear effector cell fractions obtained by 1 × G velocity sedimentation. SCMC (mean ± S.E.M. of eight different donors) is measured on T-24 (□——□), NKI-1 (■——■), and NKI-10 (△——△). 4×10^5 effectors were added per well. SCMC on K562 (●——●) and MOLT-4 (▲——▲) is measured in a 4-hr ^{51}Cr release assay (mean ± S.E.M. of five and four different donors, respectively). The effector to target cell ratio was 20:1. ADCC (mean ± S.E.M. of five different donors) is measured on antibody-coated Chang liver cells. 2×10^5 effector cells were added per well (○——○).

10, and NKI-1 targets, which grow in monolayers. However, in a 4-hr ^{51}Cr release assay, these LF cells were perfectly capable of lysing K562 and MOLT-4 cells, which grow in suspension, and showed in addition ADCC activity against Chang liver cells that was comparable with the corresponding UL. An increase in SCMC on T-24, NKI-1, and NKI-10 was observed with fraction 2 cells, which contained an average of 2% monocytes, whereas SCMC on K562 and MOLT-4 cells and ADCC on Chang liver cells remained unchanged. Maximal SCMC (which was generally higher than obtained with UL) was observed on T-24, NKI-1, and NKI-10 cells with fraction 3 cells containing an average of 16% monocytes. The same fraction 3 cells also caused a slight increase in SCMC on K562 and MOLT-4 and in ADCC. SCMC on T-24, NKI-1, and NKI-10 dropped considerably with fraction 4 cells, which contained 48% monocytes. The decrease in SCMC on K562 and MOLT-4 and ADCC was less. Finally, the MF (85% monocytes) showed no to very weak cytotoxic activity on all types of target cells, indicating that monocytes are not effective as effectors in SCMC and ADCC. Whether the SCMC of the fractions 2, 3, and 4 is caused by the presence of monocytes in these fractions or is mediated by monocyte-independent cytotoxic lymphocytes remains to be determined.

Artificially established LF-target cell contacts by agglutinins. Timonen and Saksela (27) have demonstrated that effector-target cell contact finally resulting in lysis is required for SCMC. We have previously shown that in Takasugi-Klein type of microcytotoxicity assays (which are based on visual quantitation of the detachment of target cells after incubation with effector cells) the lack of SCMC of the LF cells probably is due to the failure of these cells to establish contact with the target cells and that LF-target cell contacts could be induced by autologous monocytes within 4 hr (23). Artificially established LF-target cell contacts by agglutinins like PHA, Con A, and WGA did not result in SCMC of the LF (21). To see whether these results could be confirmed in CRA, PHA (1 μg/ml), Con A (15 μg/ml), and WGA (5 μg/ml) were added to the LF cells. After 16 hr incubation at 37°C and 5% CO_2 in the presence of these agglutinins, efforts to wash the LF cells from the target cells failed. Very dense clusters of LF cells remained firmly attached to the target cells (results not shown). However, in spite of this intense effector-target cell contact, no increase of the SCMC of the LF was observed (Fig. 8). Addition of PHA (1 μg/ml) to the UL did not result in enhanced SCMC, indicating that no mitogen-induced cytotoxicity was generated under these test conditions (results not shown).

The effect of specific anti-human IgG antibodies on SCMC and ADCC. Since the LF cells were efficient killers in ADCC assays, it cannot be excluded that the monocyte help was mediated via naturally occurring cytophilic antibodies shed from these cells during the incubation period (10–13). If such antibodies exist, they would have been present in high concentrations in the MCS, since approximately 4×10^5 monocytes were cultured in 0.25 ml volumes. Since these MCS were less effective in restoring the SCMC of the LF than relatively small percentages of intact monocytes, it seems highly unlikely that the monocyte help is mediated via this type of antibody. However, because it has been reported by others that naturally occurring antibodies mediate SCMC (10–13), we attempted to determine whether anti-human IgG antibodies could block SCMC and ADCC (against NKI-10). Various concentrations of anti-human IgG antibody were added to the SCMC and ADCC assays. In addition, the effector and target cells in SCMC and ADCC were preincubated with this reagent. The results are shown in Table II. Preincubation of the effector cells with

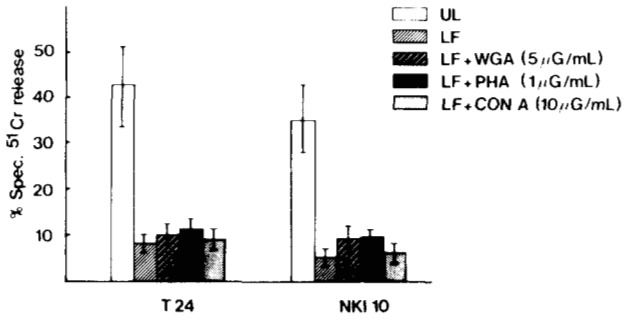

Figure 8. The effect of agglutinin-induced LF to target cell contact on SCMC (mean ± S.E.M. of two different donors).

TABLE II

The effect of specific anti-human IgG antibodies on SCMC and ADCC

	NKI-10							
	+ Anti-HLA-A_2	− Anti-HLA-A_2	T-24	NKI-1	Gil[a]	Sto[a]	K562	MOLT-4
	% inhibition of SCMC or ADCC							
Lymphocytes Pretreated[b] with $F(ab')_2$ Anti-human Fab (11 mg/ml)								
1:11	−18 ± 7[d]	−26 ± 9	9 ± 7	3 ± 7	−7 ± 2	−6 ± 7	−11 ± 5[d]	−16 ± 8[d]
1:22			4 ± 6	−2 ± 8	−20 ± 14[e]	−19 ± 20[e]		
1:88			2 ± 11	6 ± 3	−6 ± 4	−6 ± 4		
$F(ab')_2$ Anti-human Fab Present in Test								
1:11	113 ± 15	14 ± 8	18 ± 3	18 ± 5	18 ± 6	19 ± 6	5 ± 7	−8 ± 6
1:22	103 ± 40	1 ± 6	20 ± 0	18 ± 1	18 ± 2	16 ± 5		
1:44	NT	NT	5 ± 1	12 ± 2	4 ± 10	9 ± 3		
1:88	54 ± 28	10 ± 4	7 ± 15	14 ± 5	9 ± 9	7 ± 4		
$F(ab')_2$ Anti-rabbit IgG (10.2 mg/ml)								
1:10	14 ± 5	6 ± 8	10[f]	16 ± 7	15 ± 7	11 ± 2	2 ± 5	−6 ± 7
1:20	8 ± 8	18 ± 9	10[f]	15 ± 13	11 ± 2	13 ± 8		
1:80	11 ± 5	2 ± 5	2[f]	9 ± 1	8 ± 8	4 ± 6		

[a] Spontaneous ^{51}Cr release ± S.D. of Gil and Sto was 20 ± 3% and 23 ± 4%, respectively.

[b] Lymphocytes were preincubated for 45 min at 37°C, washed three times with culture medium, and tested. 4×10^5 effectors were added per well. Pretreatment of the effectors in ADCC against Chang cells at anti-human IgG dilutions of 1:22 and 1:88 resulted in an inhibition of ADCC of 16 ± 13% and 5 ± 1%, respectively (mean ± S.D. of 2 experiments). Furthermore, the sensitization of the Chang cells with rabbit anti-Chang antibodies was not inhibited by anti-human IgG antibody. Inhibition of ADCC activity with a 1:22 and 1:88 dilution of the reagent in one experiment was 4% and 8%, respectively.

[c] Mean ± S.D. of two experiments.

[d] All results with NKI-10, K562, and MOLT-4 targets are the mean ± S.D. of three experiments.

[e] Mean ± S.D. of three experiments.

[f] Results of one experiment.

various dilutions of the anti-human Ig antibody (antibody concentrations 0.125 to 1 mg/ml) for 45 min at 37°C followed by three washings did not inhibit SCMC. Slight enhancing effects were even observed on both neuroblastoma cell lines (Gil and Sto). These results indicate that the cytotoxic effects of SCMC effector cells are not mediated by antibodies with anti-target cell reactivity bound to the effector cell and are in agreement with those of other groups (12–14). Preincubation of the effectors before the ADCC tests also did not inhibit cytotoxicity, confirming that no anti-human Fc receptor activity was present in the anti-human IgG reagent used. Preincubation of the target cells with anti-human IgG antibody (45 min at 37°C) had no effect on SCMC, whereas no ADCC activity on Chang cells and NKI-10 cells could be induced by this reagent.

The presence of various concentrations of anti-human IgG antibody during the SCMC assay on monolayer targets resulted in some instances in a slight inhibition of SCMC in the two highest antibody dilutions tested (1 and 0.5 mg antibody/ml), which probably was not specific, since $F(ab')_2$ fragments of goat anti-rabbit IgG in the same protein concentration showed also slight inhibition of SCMC. The SCMC against K562 and MOLT-4 was not affected by anti-human IgG. However, ADCC activity against NKI-10, which was superimposed on SCMC, was completely blocked by antibody concentrations of 1 and 0.5 mg/ml and partially by concentrations of 0.125 mg/ml. In contrast, SCMC against NKI-10, simultaneously measured in the same experiment, was not affected by the presence of anti-human IgG. These results support the conclusion of Kay *et al.* (14) that there is no clear evidence that antibodies play a role in SCMC. In addition, the monocyte help cannot be attributed to the induction of ADCC mediated by the transfer of cytophilic antibodies from the monocytes and is probably mediated by other soluble factors.

DISCUSSION

The results of the present study indicate that lymphocytes depleted of monocytes by velocity sedimentation at 1 × G lose their SCMC. The SCMC of the highly purified lymphocytes in the LF could be restored by autologous monocytes. Reconstitution with 4 to 8% monocytes resulted in significant SCMC, whereas approximately 15% autologous monocytes were sufficient to restore the SCMC of the LF to the level achieved with the corresponding UL. Reconstitution with 15 to 30% monocytes resulted in a maximal restoration of SCMC, which was generally higher than the SCMC of the UL. Addition of >30% monocytes resulted in submaximal SCMC and indicates that certain lymphocyte-monocyte ratios are optimal for the restoration of SCMC. Optimal lymphocyte-monocyte ratios may also explain the maximal SCMC measured with fraction 3 effectors. The restoration of SC could not be attributed to helper effects of the contaminating lymphocytes in the MF, since MF cells pretreated with carrageenan were ineffective in the restoration of SCMC. In addition, monocytes could be replaced (but not optimally) by supernatants of unstimulated monocyte cultures, which also indicates that the induction of SCMC is mediated by monocytes and not by the contaminating lymphocytes. These results confirm previous findings with a Takasugi-Klein type of microcytotoxicity assay (MCA), which is based on visual quantification of target cell detachment (23).

In comparing the two types of assays, we observed, however, that the concentrations of monocytes required to restore the SCMC to the level achieved with the UL was less in the MCA than in the CRA by a factor of two. This difference is probably related to the relatively high local effector cell density in the MCA, which may permit better monocyte-lymphocyte interaction.

Our results demonstrate that very low concentrations of monocytes suffice to facilitate SCMC. Conventional monocyte-depletion procedures, including adherence to plastic surfaces and nylon wool columns or the carbonyl iron-magnet method, never result in complete monocyte removal, and therefore may partly or even completely obscure the monocyte dependency of SCMC (23).

The majority of the effectors isolated by 1 × G sedimentation require monocyte help, since the LF represents 60% of all lymphocytes, and approximately 70% of the SCMC capacity of the UL was potentially recovered in the LF. However, it remains to be determined whether this is also the case for the lymphocytes in fractions 2, 3, and 4, representing 30% of the SCMC capacity of the UL, since it may be possible that monocyte-dependent and monocyte-independent SCMC occur simultaneously.

The spontaneous cytotoxic effector cell in the ^{51}Cr release assay against monolayer targets had surface marker characteristics identical to the spontaneous cytotoxic effector in the MCA described previously (23) and is characterized as a non-Esh rosette-forming, sIg-negative, Fc receptor-bearing lymphocyte (Fig. 5). These results are in contrast with those of West *et al.* (8), who reported that the effector in SCMC was a Fc receptor-bearing cell with low affinity receptor for Esh. Our rosette procedure carried out at room temperature appeared to be equivalent or slightly superior to the Esh rosetting procedure described by West *et al.* (8). The percentage Esh rosette-forming cells was slightly higher, and more Esh were bound per lymphocyte. In addition, the Esh rosettes were more stable, since better depletions were obtained after centrifugation over Ficoll-Hypaque solutions. The lack of SCMC of the Esh rosette-forming cells could not be attributed to the NH_4Cl/Tris buffer used for lysis of the Esh, since Esh rosette-forming cells freed of Esh by incubation and vortexing at 37°C and separated by 1 × G sedimentation were equally noncytotoxic. The discrepancy is therefore probably related to the different types of target cells (K562 *vs* cells growing in monolayers) and test systems (4 hr *vs* 16 hr CRA) used. That different cytolytic mechanisms depending on the type of target cell are playing an important role in SCMC assays is illustrated by the fact that LF cells showed SCMC on K562 and MOLT-4 cells in the absence of monocytes in 4 hr CRA. These results are consistent with the findings of Timonen and Saksela (27), who reported that depletion of lymphocytes that were cytotoxic for fetal fibroblasts growing in monolayer cultures did not result in a reduction of SCMC against lymphoma (RAJI) and leukemia cells (MOLT-4, K562) growing in suspension cultures. However, it remains to be determined whether the monocyte-independent SCMC on K562 and MOLT-4 is related to the leukemic origin of these cells, or whether K562, MOLT-4, and monolayer targets are lysed by the same or different Fc receptor-bearing effector cells (one having low affinity for Esh and the other not).

The cells in the LF were not cytotoxic on target cells growing in monolayers in spite of the presence of Fc receptor-bearing cells. However, that these Fc receptor-bearing lymphocytes in the LF were the actual effectors in SCMC was illustrated by experiments in which the Fc receptor-bearing cells were removed from the LF before monocyte reconstitution. Addition of monocytes to LF cells depleted of Fc receptor-bearing cells resulted in insignificant restoration of SCMC.

On the other hand, we demonstrated that LF cells were efficient killers in ADCC in the absence of monocytes. Depletion of Fc receptor-bearing cells resulted in a strong abrogation of ADCC, and it is reported by Pape *et al.* (28) that ADCC is mediated via the Fc receptor itself. Whether this is also the case in SCMC remains to be determined. It is, however, not unlikely that the presence of the Fc receptor on the effector cell is a necessary but insufficient condition for SCMC, since Bolhuis *et al.* (29) reported that stripping of the Fc receptor from the Fc receptor-bearing cells did not affect the SCMC of these cells.

Since some groups have reported that antibodies play a role in SCMC and that SCMC therefore actually is a form of ADCC (10–13), and particularly since the LF cells in our system were cytotoxic in the presence of specific antibodies to target cells (ADCC), the possibility must be considered that the monocyte help is mediated via antibodies and actually reflects a form of ADCC. During the incubation period with target cells in the SCMC assay, lymphocytes in the presence of monocytes may produce and release small amounts of antibody that could induce ADCC. This was suggested as the mechanism for SCMC by Blair and Lane (30) and by Troye *et al.* (12). In addition, it could be possible that monocyte help reflects ADCC mediated by naturally occurring cytophilic antibodies transferred from the monocytes (10–13). Such antibodies ought to be present in high concentrations in MCS. Since MCS (derived from 4×10^5 monocytes cultured in 0.25 ml medium) are less effective than low concentrations of monocytes in the restoration of SCMC by the LF, it seems unlikely that antibodies are involved in our SCMC system.

This conclusion was confirmed by inhibition studies with anti-human IgG antibodies. Preincubation of the effector cells with this reagent had no effect on SCMC, indicating that SCMC was not mediated via antibodies with anti-target cell activity bound to the effector cell. Furthermore, the continued presence of anti-human IgG antibodies in the assay resulted in some instances in only minimal inhibition of SCMC against monolayer targets by very high antibody concentrations (1 mg and 0.5 mg purified antibody/ml), whereas SCMC against K562 and MOLT-4 was not affected. The reason for the slight inhibition by anti-human IgG antibodies present during the SCMC assay is unclear but may very well be caused by factors other than specific inhibition of antibody activity, since the same concentrations of $F(ab')_2$ fragments of goat anti-rabbit IgG, which did not cross-react with the anti-human IgG, showed comparable effects on SCMC. In contrast, ADCC against NKI-10, which was superimposed on the SCMC against these targets, was completely blocked at antibody concentrations of 1 and 0.5 mg/ml and partially at concentrations of 0.125 mg/ml. The SCMC, however, was unaffected by our anti-human Ig reagent. These data suggest that the monocyte help is not mediated by antibodies and that antibodies probably are not involved in SCMC. These data are consistent with the findings of Kay *et al.* (14). In contrast, other investigators reported that a significant part of SCMC actually reflects ADCC induced by natural antibodies (12, 13). The discrepancies between these and our results may very likely be related to the purity of and differences in the anti-human IgG reagents (13, 14).

It is more likely that the induction of SCMC by monocyte culture supernatants is mediated by non-Ig soluble factors, like those described by Peter *et al.* (31) and Koide and Takasugi

(32). Recently, Trinchieri *et al.* (33, 34) have reported that SCMC can be augmented considerably by interferon. Studies to investigate whether our MCS contain interferon activity or whether they induce interferon synthesis by LF cells are in progress at the moment.

We have demonstrated previously that the lack of SCMC of the LF might be due to the failure of these cells to make proper contact with the monolayer target cells (23). Such contacts, which are required for target cell killing by specific cytotoxic T cells (35) are probably also necessary for SCMC (27). LF-target cell contacts followed by lysis of the target cells could be restored by addition of monocytes (23). We find that artificial LF-target cell contact induced by agglutinins does not result in SCMC (Fig. 8). This suggests that the spontaneously cytotoxic, Fc receptor-bearing effector cell may be recognizing certain membrane determinants present on a large variety of cultured target cells in a specific way. We hypothesize that this recognition might be mediated by an additional receptor site on the Fc receptor-bearing lymphocyte, which is induced or unmasked only in the presence of monocytes or a soluble monocyte product. In contrast, the leukemias K562 and MOLT-4 that are maintained in suspension cultures probably possess membrane structures that allow direct contact with the effector cells (without any monocyte help). The possibility that different types of effector cells are involved in the killing of target cells growing in monolayer or in suspension cultures may not, however, be excluded.

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