COOPERATIVE EFFECTS OF COLONY-STIMULATING FACTOR 1 AND RECOMBINANT INTERLEUKIN 2 ON PROLIFERATION AND INDUCTION OF CYTOTOXICITY OF MACROPHAGE PRECURSORS GENERATED FROM MOUSE BONE MARROW CELL CULTURES

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Cells of the monocyte-macrophage lineage are among the most important effectors of natural as well as lymphokine-mediated cytotoxicity against tumor cells (1), microbial and fungal targets (2, 3), and protozoa like *Leishmania donovani* (4). These effector functions, however, are not only a feature of mature macrophages. We recently described a nonadherent and nonphagocytic macrophage precursor cell that is able to spontaneously and extracellularly kill a broad pattern of target cells, ranging from YAC-1 lymphoma cells to *Candida albicans* yeast phase microorganisms to protozoa of the genus *Leishmania* (5). These macrophage precursors can be isolated and purified from spleen and liver of normal mice, and of mice in which inflammatory conditions had been induced (6, 7). Macrophage precursors endowed with fungicidal, leishmanicidal, and NK activity could also be reproducibly generated in 3-d-old bone marrow liquid cultures supplemented with L cell-conditioned media as a source of CSF-1 (8–10). In keeping with results from other laboratories (11), the NK activity of these cells was somewhat lower than that exerted by their organ-derived counterparts. We also met with different success when we attempted to boost this activity with human rIL-2, whereas the lymphokine was very effective in augmenting the NK activity of organ-derived macrophage precursors (12). The bone marrow–derived macrophage precursors were able to transiently proliferate in IL-2-containing media and were shown to express the p75 component of the IL-2-R (13). This body of evidences suggested a close relationship between cells of the monocyte-macrophage lineage and NK cells. A major problem was represented by the difficulty to reproducibly obtain homogenous populations endowed with NK activity from the 3-d old bone marrow liquid cultures. In this paper we describe a modification of our bone marrow liquid culture, using fractionated bone marrow cells as a starting population. The initial fractionation is performed to enrich for precursor cells of NK ac-

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tivity. This technique enabled us to generate large numbers of macrophage precursors exerting strong NK activity from bone marrow cultures supplemented with human recombinant (hr)[1] IL-2- and L cell-conditioned media or hrCSF-1. These cells had the morphology of large granular lymphocytes (LGL), coexpressed the surface markers NK 1.1 (14), typical of NK cells and F4/80 (15), specific for cells of the monocyte-macrophage lineage, and differentiated to mature phagocytes under the influence of CSF-1. Furthermore, the availability of recombinant material allowed us to dissect the roles of the two growth factors (IL-2 and CSF-1) in the generation of bone marrow-derived, NK-active macrophage precursors.

Materials and Methods

Mice. 6–8-wk-old inbred C57/BL6 (H-2^b) mice were obtained from the Zentrale Versuchstieranstalt, Hannover, FRG, and were maintained in a pathogen-free environment for at least 2 d before use.

Media. RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 15% FCS, glutamine (5 mM), 100 U/ml penicillin, and 1 μg/ml streptomycin (hereafter referred to as complete medium), was used throughout the study.

Factors. Purified human rIL-2 from *Escherichia coli* (specific activity of 2×10^7 U/mg protein) was kindly provided by Dr. Conradt, GBF, Braunschweig, FRG. Purified human rCSF-1, was a kind gift from Dr. Peter Ralph, Cetus Corp., Emeryville, CA. L cell-conditioned medium, prepared in our lab as a 4-d supernatant of fibroblasts from a L-929 cell line, was used as a source of mouse CSF-1.

Antibodies. F4/80 rat anti-mouse macrophage mAb (IgG2b) (15), a kind gift of Dr. Siamon Gordon, Sir William Dunn School of Pathology, University of Oxford (Oxford, UK), was provided as dialyzed ammonium sulphate precipitate from tissue culture supernatant, and was used at a 1:20 dilution. NK-1.1 mouse anti-mouse NK cells mAb (IgG2b) from the hybridoma line PK136 (14) (purchased from American Type Culture Collection, Rockville, MD), was provided as tissue culture supernatant, and was used at a 1:2 dilution.

Anti-Lyt-2.2 mouse anti-mouse cytotoxic lymphocyte mAb (IgM) was obtained from Cedarlane Laboratories Ltd., Canada. Leu-M3 mouse anti-human monocyte and macrophage mAb (IgG2b) (No. 7490; Becton Dickinson Monoclonal Center, Mountain View, CA) was used as the isotype negative control. Fluorescein-conjugated affinity pure F(ab)'2 fragment goat anti-rat IgG (Code No. 112-016-062) and goat anti-mouse IgG (Code No. 115-016-062) were purchased from Dianova GmbH, Hamburg, FRG. 10 μg/ml human Ig solution was prepared by diluting 5 g Ig7 S human intravenös (from Serapharm 4400 Münster, Zul. - Nr. 242a/1b) with PBS + 2% newborn calf serum + 0.01% (wt/vol) NaN_3. It was used to preincubate the cells before fluorescent staining.

Tumor Cell Lines. YAC-1, a Moloney virus-induced lymphoma of A/Sn origin and P815, mastocytoma of DBA/2 origin, were maintained as tissue cultures in RPMI 1640 medium supplemented with 10% FCS and 0.1% gentamicin.

BM Culture. BM cells were collected from the marrow of two femurs of each mouse. The marrows were dispersed gently with a syringe with a 0.8-mm diameter needle, washed once, then resuspended in RPMI + 10% FCS at a cell density of 3×10^7 cells/ml.

The cell suspension was then fractionated through a discontinuous Percoll gradient. Cells from the upper four fractions were pooled and washed with RPMI three times. 10^7 cells in 10 ml of complete medium were plated in 9-cm Petri dishes (Grenier, Nürtingen, FRG). 15% L cell-conditioned medium (as a source of CSF-1) and rIL-2 at a concentration of 100 U/ml were added to the cultures. The cultures were maintained at 37°C in 5% CO_2 for 3 d or more.

Fractionation of Cells through a Discontinuous Percoll Gradient. A 60.6% Percoll solution (Osmolarity 285 m OsM/kg H_2O) was diluted in RPMI 1640 with 10% FCS to the following

[1] *Abbreviations used in this paper:* hr, human recombinant; LGL, large granular lymphocytes.

five different concentrations: 55.0%, 50.0%, 45.3%, 40.8%, and 30.0%. 2 ml of each solution was carefully layered in a plastic conical tube (model 2099; Falcon Labware, Oxnard, CA) with 2 ml 60.6% solution at the bottom. Cells in 2 ml RPMI + 10% FCS were placed on top of the gradient and the tube was spun at 550 *g* for 30 min. Cells were collected with a syringe as fraction 1, 2, 3, 4, 5, and 6 from the top and washed three times with RPMI 1640.

^{51}Cr Release Assay against Tumor Cells. Effector cells in triplicate round-bottomed 96-well (Nunc, Roskilde, Denmark) microtiter wells were incubated with 5×10^3 labeled tumor cells (200 μCi/5×10^6 cells for 1 h at 37°C in 0.5 ml RPMI 1640) in 200 μl complete medium at 37°C in 5% CO_2 for 4 h. At the end of the incubation period, an aliquot of the supernatant was collected and its radioactivity was measured in a gamma counter (MR480 Automatic Gamma Counting System, Kontron Analytical, Everett, MA). The spontaneous release (SR) was determined by incubation of the target cells without effectors. The percentage of specific lysis was then calculated by using the formula: percent specific lysis = 100 × [(cpm experimental release − cpm spontaneous release)/(cpm total releasable − cpm spontaneous release)]. Total releasable counts from tumor cells were obtained before lysis in 1 N HCl.

Proliferation Assay. [^{3}H]dThd incorporation into replicating cells was measured to determine the proliferative ability of the effector cell populations. 5×10^3 cells/well in 200 μl complete medium were seeded into flat-bottomed microtiter plates (model 3072; Falcon Labware), and incubated with or without the factors at 37°C for different time periods. 1 μCi/well of [^{3}H]dThd was then added, followed by another 18 h of incubation. The cells were then harvested by a suction filtration apparatus (Skatron, Inc., Sterling, VA). The [^{3}H]radioactivity bound to fiberglass filters was finally determined by β scintillation counting (LS 1801; Beckman Instruments, Inc., Fullerton, CA).

Microculture of the Effector Cells. The cells from BM bulk cultures or fresh BM were cultured at a density of 10^5 cells/200 μl in 96-well round-bottomed microtiter plates (Nunc). Culture medium was complete medium. According to the purposes of studies, hrIL-2, hrCSF-1, or L-conditioned medium (as source of mouse CSF-1) were added either separately or concomitantly into test wells. Before cytotoxicity assay (^{51}Cr release from tumor cells), the cells in microcultures were washed twice and the labeled target cells were added. Assays were set up in triplicate.

Fluorescence Staining and Flow Cytometry Analysis. $0.5–1 \times 10^6$ cells were suspended in 40 μl PBS supplemented with 2% newborn calf serum and 0.01% (wt/vol) NaN_3 (PBS-NCS). Cells were first mixed with 40 μl human Ig solution (10 μg/ml) for 15 min on ice, washed once, and then incubated either in 40 μl medium alone or with the 40 μl mAb solution for 45 min on ice. Cells were washed thrice with PBS-FCS and stained with the relevant FITC goat antibody (40 μl; 1:40). After 30 min on ice, cells were washed again thrice and analyzed on a flow cytometer (FACSTAR; Becton Dickinson & Co., Mountain View, CA). For each test fluorescence signals of 2×10^4 viable cells were accumulated on a logarithmic scale into list mode data files by a Consort 30 computer system.

Results

Enrichment of Bone Marrow Progenitor Cells for Anti-YAC-1 Activity in the Light Fraction of a Discontinuous Percoll Gradient. It is known that mouse fresh bone marrow cells are the source for progenitor cells of NK activity and that cultivation of fresh bone marrow cells in IL-2-containing medium results in the development of YAC-1 lytic cells (16–18). To enrich for such precursor cells we fractionated fresh bone marrow cells on a discontinuous Percoll gradient. All fractions were cultivated in IL-2 and tested after 24 or 48 h for YAC-1 lytic activity. By far the highest activity was obtained with cells of the light fraction (Fig. 1). The induction of the lytic function was totally dependent on incubation with IL-2 and no lytic function developed in the absence of it.

Proliferation of Light Fraction Bone Marrow Cells in Medium Containing CSF-1. Cultivation of light Percoll fraction 1-4 in IL-2 did not result in any significant prolifera-

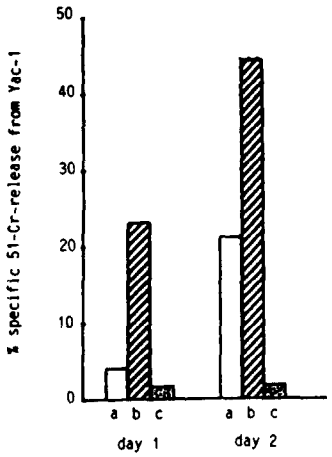

FIGURE 1. Bone marrow IL-2-inducible YAC-1 lytic cell progenitors reside mostly in light Percoll fractions. NK activity of (*a*) total bone marrow cells; (*b*) cells from Percoll fraction 1-4; and (*c*) cells from Percoll fraction 5-6. Tests were carried out after the cells had been incubated with 100 U/ml rIL-2 in microculture for 1 or 2 d. 2.5×10^5 cells were seeded into each microtiter well in 200 μl at the start of culture. 5×10^3 labeled YAC-1 targets were put into each test well for the ^{51}Cr release assay.

tion. In contrast, cultivation in medium containing CSF-1 produced a strong proliferative response (Fig. 2). However, cells cultivated and proliferated in CSF-1 had virtually no lytic activity. Even when the CSF-1 proliferated cells were refractionated on a second Percoll gradient, no significant lytic activity was obtained in any of the fractions.

Cultivation of F1-4 Bone Marrow Cells in IL-2 Plus CSF-1 Results in the Induction of Actively Proliferating Macrophage Precursors Endowed with NK Activity. Cultivation of F1-4 in a combination of CSF-1 (100 U/ml rCSF-1 or 10% L cell-conditioned medium) and IL-2 (100 U/ml) caused active cellular proliferation accompanied by the onset of a strong NK activity. The development of the latter activity coincided with the appearance in the culture of cells with the morphology of LGL (Fig. 3, *b* and *c*). After 3 d of culture in the growth factor cocktail, cells were harvested and fractionated once more on a Percoll gradient. The NK activity was again concentrated in the fraction 1-3 (Fig. 4), which at this time of the culture represented a homogenous population of macrophage precursors, that could be cloned in soft agar in the presence of CSF-1 as the only growth factor and matured to typical macrophages as described previously (9). These cells will be hereafter referred to as BM-derived macrophage precursors. Fraction 4-6 contained mature granulocytes, derived from precursors that had also been present in F1-4 of the first Percoll gradient.

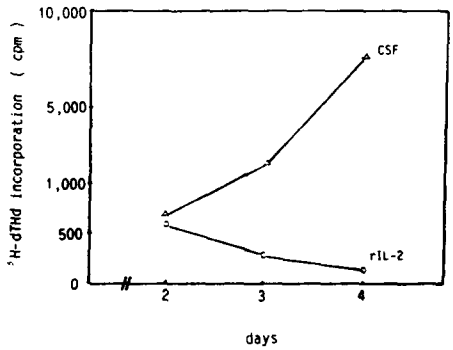

FIGURE 2. Fraction 1-4 bone marrow cells, containing YAC-1 lytic cell progenitors, depend on M-CSF containing medium for optimal proliferation. 5×10^3 cells in 200 μl were plated in flat-bottomed microtiter (96-well) plates and incubated with (O) rIL-2, 100 U/ml or (Δ) CSF, 10% L-conditioned medium. [^{3}H]dTHd incorporation was assayed on various days after incubation.

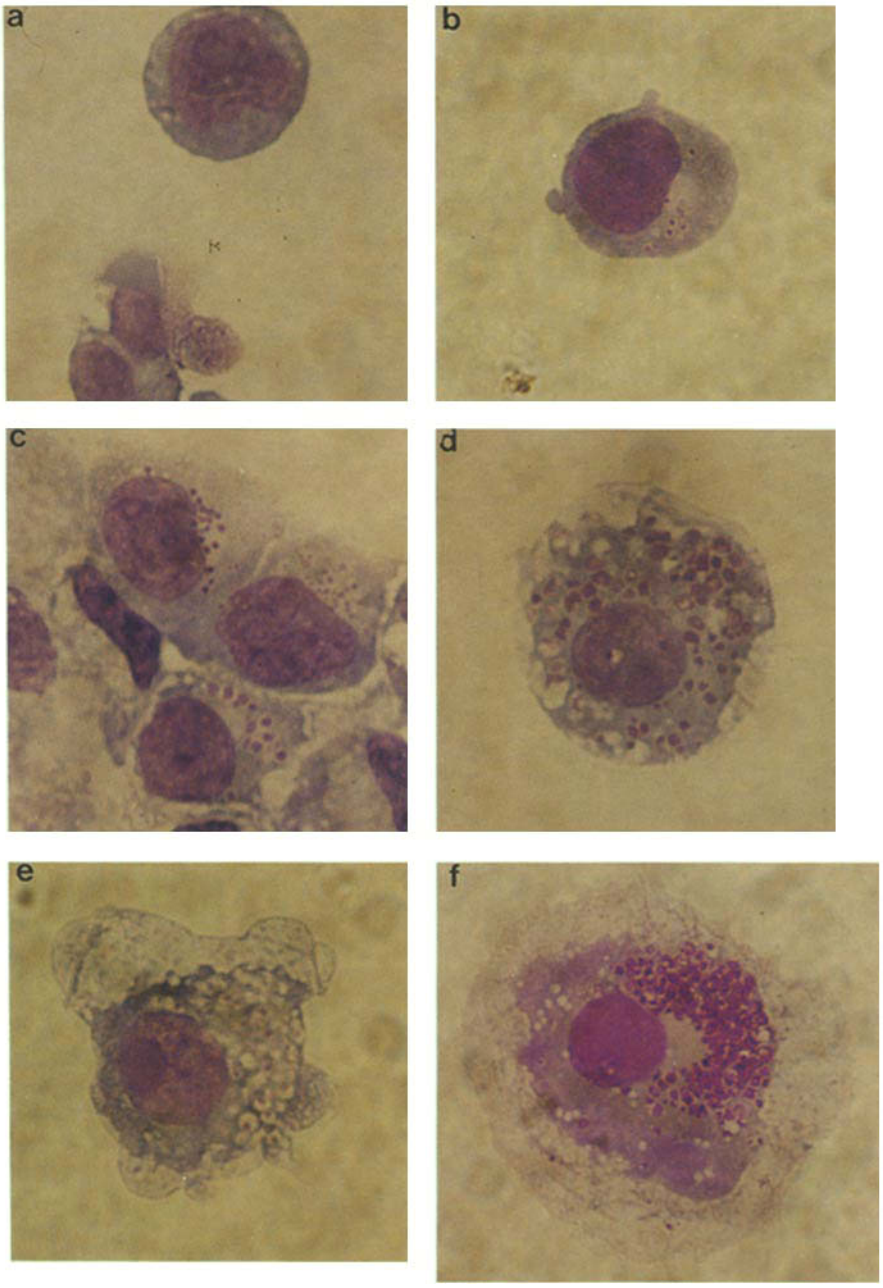

FIGURE 3. (*a*) Macrophage precursor from a 3-d culture in only CSF-1. No formation of granula, no anti-YAC-1 activity. (*b*) Macrophage precursor from a 3-d culture in CSF-1 plus IL-2. Granula formation, anti YAC-1 activity. (*c*) Macrophage precursors from a 4-d culture in CSF-1 plus IL-2. More granula have developed. (*d*) Macrophage precursor from a 5-d culture in CSF-1 plus IL-2. Pronounced granula formation. Strong anti-YAC-1 activity. (*e*) Macrophage precursor from a 5-d culture in CSF-1 plus IL-2, further cultivated for 48 h in only CSF-1. Maturation, cleaning of granula, and loss of anti YAC-1 activity. (*f*) Macrophage precursor from a 5-d culture in CSF-1 plus IL-2, further cultivated for 48 h in only IL-2. Excessive granula formation and high anti-YAC-activity.

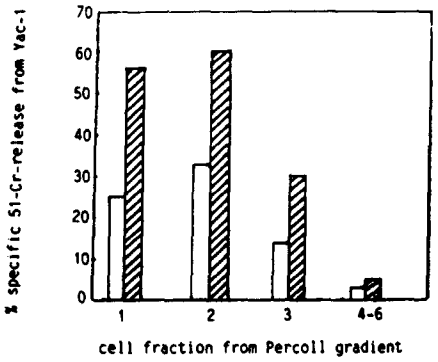

FIGURE 4. The YAC-1 lytic cells from 3-d rIL-2 (100 U/ml) + CSF-1 (10% L-conditioned medium) bulk culture reside in light Percoll fractions. ^{51}Cr release assays were performed for (□) 4 h, and (▨) 8 h the E/T ratio of 40:1.

Survival and Proliferation of BM-derived Macrophage Precursors as well as Their NK Activity Depend on the Presence of Both Growth Factors. BM-derived macrophage precursors, isolated as described above, were further cultivated in media supplemented by either rIL-2 (100 U/ml) or L cell-conditioned medium (10%), or a combination of both. hrCSF-1 (100 U/ml) was used in selected experiments to exclude the possibility that the effects seen in the presence of L cell-conditioned medium were due to other contaminating soluble factors. BM-derived macrophage precursor cells were either transferred to flat-bottomed microtiter plates (5×10^3 cells/well) to test their proliferative activity under the influence of different growth factors, or seeded into round-bottomed microtiter plates (10^5 cells/well) and supplied with different growth factors to analyze the kinetics of induction of NK activity.

As shown in Fig. 5, only CSF-1 turned out to be the appropriate growth factor to sustain cell proliferation. In the presence of IL-2 alone, the cells gradually lost their proliferative potential. Addition of IL-2 to CSF-1 had no influence on the proliferative activity of BM-derived NK cells.

Cells kept in CSF-1 alone, however, soon lost their NK activity completely, underwent further proliferation, and finally developed to mature phagocytes. IL-2 alone was sufficient to maintain good NK activity, but the combination of CSF-1 and IL-2 yielded by far the best results (Fig. 6). As CSF-1 did not seem to stimulate the NK activity of BM-derived NK cells, the enhancement of cytotoxicity observed was apparently due to an increase in the effector cell number.

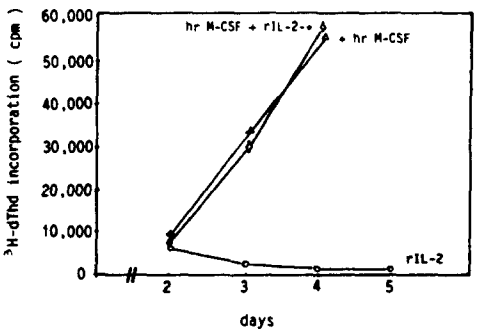

FIGURE 5. Growth factor test: proliferation of YAC-1 lytic cells of Percoll fraction 2 from 3-d rIL-2 + CSF-1 bulk culture. 5×10^3 cells in 200 μl were seeded in 96-well flat-bottomed microtiter wells and incubated with: (○) rIL-2 (100 U/ml); (△) hrCSF-1 (100 U/ml); and (◇) rIL-2 (100 U/ml) + hrCSF-1 (100 U/ml). [^{3}H]dTHd incorporation was assayed on various days after incubation.

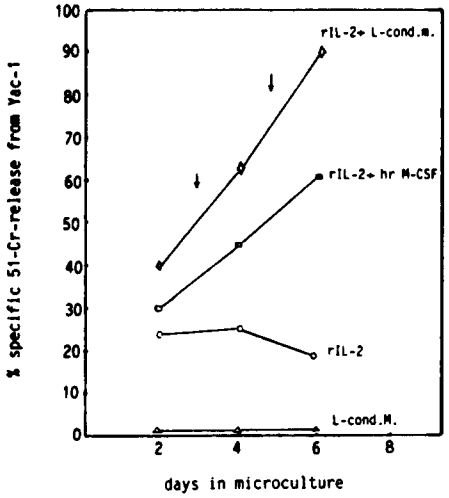

FIGURE 6. Kinetics of YAC-1 lytic activities of fraction 2 cells from 3-d rIL-2 + CSF-1 bulk culture. 10^5 cells were further cultured in 200 μl medium in round-bottomed 96-well microtiter plates and assayed on various days of this microculture. Culture conditions were: (○) rIL-2 (100 U/ml), (◇) rIL-2 (100 U/ml) + CSF-1 (10% L-conditioned medium), (□) rIL-2 (100 U/ml) + hrCSF-1 (100 U/ml), and (△) M-CSF-1 (10% L-conditioned medium). Cells were washed three times before the ^{51}Cr release assay and then incubated with 5×10^3 labeled YAC-1 targets for 4 h. Arrows indicate the day of feeding.

Peak NK activity was reached after 5 d in culture with media containing IL-2 plus CSF-1. The strongly lytic effector cells were then transferred to microcultures supplemented with either of the growth factors, cultured for additional 24 h, and subsequently tested for NK activity. The cultures supplemented with IL-2 alone maintained a constantly high NK activity paralleled by an increase in the content of cytoplasmic granules revealed by microscopical analysis of Giemsa-stained slides (Fig. 3 *f*). These granules kept increasing over an additional 4–5 days of culture (Fig. 3 *f*), at the end of which the cell recovery started decreasing dramatically. In contrast, when the cells were transferred to cultures supplemented exclusively with CSF-1, they lost their NK activity within 24 h (Fig. 7). This loss of activity correlated well with the loss of cytoplasmic granules (Fig. 3 *e*). Cells retained their proliferative activity and finally matured to macrophages.

Only when BM-derived NK cells were kept in media containing both growth factors could their proliferative and lytic activity be maintained.

Target Selectivity of Effector Cells. To demonstrate that the lytic activity of BM-derived NK cells was restricted to the cells of the NK-sensitive lymphoma YAC-1, the cells

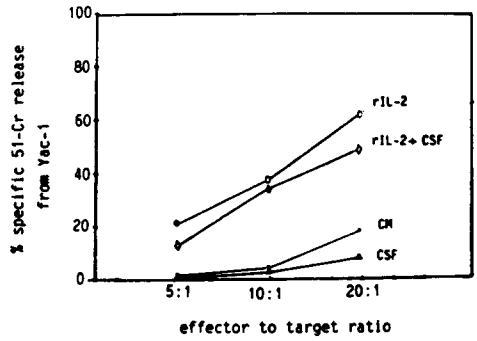

FIGURE 7. Variation of the cytotoxicity of the YAC-1 lytic cells from 5-d rIL-2 + CSF-1 bulk culture when transferred to different culture conditions: (○) rIL-2 (100 U/ml) alone; (△) CSF (10% L-conditioned medium; (●) CM (complete medium); and (△) CSF (10% L-conditioned medium); (●) CM (complete medium); and (◇) rIL-2 (100 U/ml) + CSF (10% L-conditioned medium). ^{51}Cr-release assay was performed 1 d after the transfer.

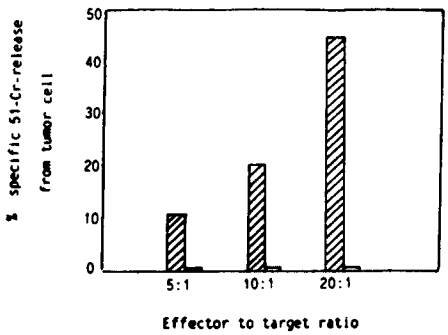

FIGURE 8. Cytotoxicity of cells of Percoll fraction 1-3 from 5-d rIL-2 + CSF-1 bulk culture in a 4-h ^{51}Cr release assay against (▨) YAC-1 and (□) P815.

were used as effectors in a cytotoxicity assay against YAC-1 and P815 mastocytoma cells as an NK-insensitive target. As shown in Fig. 8, they performed strong cytotoxicity against YAC-1 cells leaving P815 cells totally unaffected.

Analysis of the Surface Markers Expressed by BM-derived Macrophage Precursors. BM-derived macrophage precursors from a 3-d culture were analyzed for surface marker expression using mAbs against the lineage restricted antigens NK1.1 (specific for NK cells), F4/80 (specific for mature macrophages), Lyt-2.2, and L3T4. FACS analysis showed that 90% of the cells were stained positive by the anti-NK 1.1 antibody and only a marginal amount was positive for F4/80 (Fig. 9 *a*). After another 2–3 d in IL-2 plus CSF, the cells were equipped with more and larger cytoplasmic granules (Fig. 3, *b, c,* and *d*) so that their YAC-1 killing activity was very high. At this time, the cells started to significantly express F4/80 surface marker, and retained NK-1.1 marker very well (Fig. 9 *b*). FACS analysis of the 5-d cells showed >90% of the cells staining positive with NK 1.1 antibody, and ~70% of the cells staining positive for F4/80. Thus, 60–70% of the cells coexpressed F4/80 and NK 1.1. A homogenous population (100%) of cells coexpressing the two antigens could be evidenced setting the size scatter in order to gate for large granular cells (Fig. 9 *c*). The remaining NK-1.1$^+$, F4/80$^-$ cells were comparatively smaller. They were still too immature to strongly express the F4/80$^+$ marker. They matured to F4/80, NK 1.1$^+$ cells within the next 48 h.

The expression of the NK 1.1. marker was independent of the presence of IL-2 and occurred much in the same way in cultures kept in CSF-1 exclusively. NK 1.1$^+$, F4/80$^+$ BM-derived macrophage precursors did not express the Lyt-2 or the L3T4 antigens at any time of the culture (data not shown).

Morphological Analysis of BM-derived Macrophage Precursor Differentiation In In Vitro Cultures. The microscopical analysis of bone marrow-derived macrophage precursors differentiating under various condition yielded a strikingly different picture depending on the growth factor(s) used. F1-4 bone marrow cells kept exclusively in CSF-1 developed to NK 1.1$^+$ macrophage precursors that differentiated in 5–6 d to normal macrophages. No cytoplasmic granules could be observed during the whole process of proliferation and differentiation of these cells (Fig. 3 *a*).

The same cells cultured in IL-2 alone assumed during the first 3 d of cultures the appearance of typical LGL. Later on, however, these cells proved unable to proliferate and to survive in vitro; only a small percentage developed to mature, F4/80$^+$ macrophages.

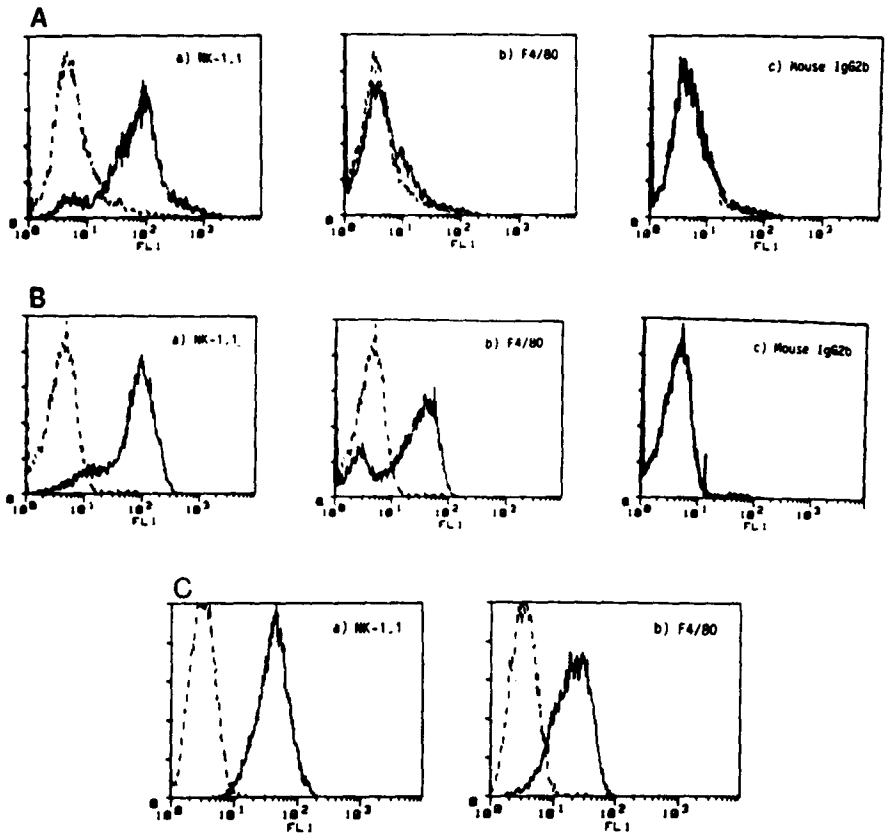

FIGURE 9. (*A*) Antigenic profile of YAC-1 lytic cells of Percoll fraction 2 from 3-d rIL-2 + CSF-1 bulk culture. (*B*) Antigenic profile of highly YAC-1 lytic macrophage precursor cells. They were obtained by further cultivating NK-1.1$^+$ F4/80$^{+/-}$ cells in rIL-2 + CSF-1 for 3 d. (*C*) By gating for large granular cells, a cell population expressing NK-1.1 and F4/80 was obtained.

In the growth factor cocktail, typical LGL (90%) appeared and underwent differentiation to macrophages (Fig. 3, *b*–d). The whole process was completed within 10–14 d. When the supernatant cells were continuously subcultured, the proliferative and lytic conditions could be maintained much longer. When CSF-1 was withdrawn on day 5 and only IL-2 (100 U/ml) was further supplied, the amount and size of cytoplasmic granules in the cells increased (Fig. 3 *f*), but the proliferation decreased and finally stopped. In contrast, when IL-2 was withdrawn on day 5 and only CSF-1 was further supplied (hrCSF-1, 100 U/ml or 10% L cell-conditioned medium) the granules disappeared (Fig. 3 *e*), the cells continued to proliferate, lost their NK activity, and finally matured to macrophages.

Discussion

Over the last 10 yr, much interest has been focussed on the origin of NK cells, on their lineage, and on their development from precursors to mature effector cells.

In the murine system, the site of origin of these cells has been mapped to the bone

marrow by the studies of Haller et al. (19) and a functional bone marrow microenvironment has also been shown to be essential for the normal development of NK cells (20).

The establishment of in vitro marrow culture systems, together with the more recent availability of purified or recombinant materials, has made it possible to study the influence of various growth factors on the proliferation and differentiation of NK cells. This approach has been used with success by our as well as by many other labs (8, 9, 16–18, 21–23). A major problem, however, has been represented by the variety of different cell types composing the starting bone marrow population. Ideally, a culture should be started with a population as enriched as possible for cells committed to developing NK activity in vitro. Attempts in this direction have been undertaken recently (16–18, 21–23).

In this paper, we report on the establishment of bone marrow liquid cultures enriched for cells committed to exert NK activity. This was achieved by fractionating the cells on a Percoll density gradient, cultivating them for 24–48 h in the presence of hrIL-2 (100 U/ml), and thereafter testing the cells for NK activity. In line with the data from other labs (16, 17) NK precursors were found to reside only in the low density Percoll fraction of the Percoll gradient and were stimulated by IL-2 to develop NK lytic activity (16, 21). NK precursor-enriched bone marrow cultures were then established using as a starting population the cells developing the best IL-2-dependent NK activity. IL-2 proved to be essential for the development of a strong lytic activity and for the generation of cells bearing cytoplasmic granula, but was unable to induce a sustained proliferative response. For this to take place, the cells had to be cultured in the presence of CSF-1. Optimal additive response (proliferation plus induction of NK activity) was obtained by supplementing the cultures simultaneously with both growth factors (CSF-1, 100 U/ml; IL-2, 100 U/ml). A second Percoll fractionation was undertaken after 3 d in culture with the growth factor cocktail, aimed to enrich for the cells responsible for the NK activity in the culture. These cells were found again in the low density fraction of the gradient, expressed the NK cell-specific marker NK 1.1 and low amounts of the macrophage-specific marker F4/80, and are hereafter referred to as BM-derived macrophage precursors. To get some more insight on the lineage commitment of the BM-derived macrophage precursors, we set out to study the effect of recombinant, lineage-restricted growth factors on this highly functionally enriched, nearly homogenous (90% of the cells are NK 1.1^+) cell population.

BM-macrophage precursors were shown to require CSF-1, either as an L cell supernatant or as recombinant molecules, for optimal survival, proliferation, and differentiation; a fact that defines them as components of the mononuclear phagocyte system (24). The NK activity of the BM-NK macrophage precursors cultivated in CSF-1 alone was very low, and their morphological appearance was that of large agranular lymphocytes (25). Lower proliferating potential, but high NK activity and a morphology typical of LGL were in contrast typical for BM-macrophage precursors grown in rIL-2 (100 U/ml). The best results, again, were obtained in a growth factor cocktail, sustaining strong proliferation of actively lytic cells (peak NK activity after 5 d of culture) and their final differentiation to macrophages. Of particular interest is the fact that the effects of the two growth factors are additive, which means that the cells coexpress both receptors and that no reciprocal down-regulation of the

receptors takes place as described in other systems using precursor cells and a growth factor cocktail. Also, the effects of the two growth factors can be reversed at any time in <24 h simply by withdrawing one of the factors. This is a very important observation that rules out the possibility that the cultures contain two or more populations, only one of which is selectively stimulated by either growth factor.

Another indication of the homogeneity of the population and of its commitment to the macrophage-monocyte lineage comes from the monitoring of the surface marker. BM-macrophage precursors supplemented with both growth factors shifted from a NK 1.1^+ (90%) $F4/80^{+/-}$ population to a virtually homogenous NK 1.1^+ population in which 60–70% turned $F4/80^+$ at day 5, when peak NK activity was observed. It should be noted at this point that the expression of the NK 1.1 antigen was not induced by IL-2, since it occurred similarly when the cells were precultured in the sole presence of CSF-1. Cells expressing NK 1.1 without concomitantly being lytic for YAC-1 cells have been described previously (20). Thus, it appears that an interplay between CSF-1 and IL-2 is necessary for the optimal generation of lytic NK cells from bone marrow liquid cultures. These culture conditions are likely to be similar to the situation in vivo in the organs, where cells come in contact with soluble factor cocktails.

IL-2 played the key role in the acquisition of NK activity by macrophage precursors. The time of growth factor addition was critical for successful stimulation. Only marginal stimulation of NK activity occurred when IL-2 was added after the cells had been precultured in CSF-1.

IL-2 has been reported not to be sufficient for long-term proliferation in vitro (26). For a limited time period macrophage precursors sustain proliferation in IL-2 alone (13) as has also been shown for highly enriched sorted splenic NK cells (27). This proliferative effect is a short-term one and is best obtained when the cells are seeded at high density and in round-bottomed wells.

The data with anti-YAC-active cells cultivated from bone marrow indicate that the relative composition of the two factors regulates the function of this cell type. In the absence of IL-2, macrophage precursors proliferate and mature to typical macrophages. When both factors are present, the cells proliferate and develop granula and spontaneous anti-YAC-activity. In the presence of only IL-2 without any CSF-1, granula formation occurs in an excessive way, and proliferative activity is lost. When cells that had been cultured in both growth factors were depleted from IL-2, they lost their granules, lost NK-like activity, and developed to normal macrophages.

Morphologically anti-YAC-activity of the cells correlated completely with the generation of granula, which depended on the presence of IL-2 in the culture. The excessive accumulation of granula in the presence of only IL-2 has so far never been observed with splenic NK cells, which may be explained by the fact that in vivo combinations of growth factors always act on the cells.

Apart from the here-reported dependance of NK-active macrophage precursors on CSF-1 as growth factor for proliferation and IL-2 for induction of NK activity, other reports have previously demonstrated functions of NK cells that are usually associated with cells of the macrophage lineage, such as IL-1 secretion (28), production of TNF-α (28), or their limited capacity to phagocytose (29).

The fact that macrophage precursors coexpress NK-1.1 and F4/80, that development of granula and anti-Yac-1 activity is induced by IL-2, and that CSF-1 is respon-

sible for proliferation and final maturation of the cells to macrophages supports the assumption that at least a major part of the NK cell compartment represents early stages of the macrophage lineage.

Summary

Precursor cells for NK activity, present in the light fraction of fresh mouse bone marrow, were cultivated in vitro in the presence of either CSF-1, IL-2, or a combination of both factors. In the presence of only CSF-1, strong proliferation was induced. Cells quickly passed the macrophage precursor stage and matured to typical macrophages. Neither granula formation nor NK activity were induced. Under culture conditions with only IL-2 NK activity had developed after 3 d, however, no significant proliferation occurred. In the presence of both factors strong proliferation was induced, and concomitantly, granula formation and NK activity developed. Apparently, proliferation depended on CSF-1 and granula formation, and NK cytotoxicity was induced by IL-2. When proliferating cells with strong anti-YAC-1 activity from a culture in CSF-1 plus IL-2 were further cultivated in only IL-2, the content of granula further increased, whereas proliferation gradually stopped. In contrast, when these cells from CSF-1 plus IL-2 culture were further cultivated in only CSF-1, granula disappeared and NK activity was lost, whereas sustained proliferation and differentiation to macrophages occurred. Only under culture conditions with both factors were proliferation and NK activity both maintained. More than 90% of cells from a 3-d culture in CSF-1 plus IL-2 expressed the NK 1.1. marker, whereas F4/80 was only marginally detected by FACS analysis. After two further days in culture, 70% of the cells expressed F4/80 and 60% coexpressed NK 1.1. and F4/80. By setting the size scatter in order to gate for large granular cells, a population was obtained with 100% coexpression of NK1.1. and F4/80. The data indicate that early cells of the macrophage lineage can develop into different functional and morphological directions depending on the varying influence of IL-2 and CSF-1.

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