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Cytotoxic functions of blood mononuclear cells in patients with colorectal carcinoma treated with mAb 17-1A and granulocyte/macrophage-colony-stimulating factor

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Summary. Unconjugated monoclonal antibodies (mAb) may induce tumour regression in patients. The mechanisms of action are complex. Antibody-dependent cellular cytotoxicity (ADCC) is considered one of the effector functions. Augmentation of the killing capacity of cytotoxic cells may thus be a way to increase the therapeutic potential of mAb. Granulocyte/macrophage-colony-stimulating factor (GM-CSF) has been shown to enhance this function in vitro. Eighteen patients with metastatic colorectal carcinoma received GM-CSF (250 μg m^{-2} day^{-1} s.c.) for 10 days and a single infusion of the anti-(colon carcinoma) mAb 17-1A (mouse IgG2A) (400 mg) on day 3 of the cycle. The cycles were repeated once a month four times. Neutrophils, eosinophils, monocytes and lymphocytes increased significantly in a biphasic way. However, at the fourth cycle the rise in white blood cells was significantly lower compared to the preceding courses. ADCC (SW948, a human CRC cell line, + mAb 17-1A) or peripheral blood mononuclear cells (PBMC) was significantly ($P < 0.05$) augmented by day 6 of a cycle and then declined gradually and, at the end of a cycle, the ADCC activity had returned to the pretreatment level. The spontaneous cytotoxicity of PBMC against the natural-killer-resistant cell line, SW948, varied in a similar way. During GM-CSF treatment there was also a significant increase in $FcRI^+$ (CD64), $FcRII^+$ (CD32), $FcRIII^+$ (CD16) and $CD14^+$ cells but not of $CD56^+$ cells.

Key words: Monoclonal antibodies – GM-CSF – Colorectal carcinoma

Introduction

Colorectal carcinoma (CRC) cells might be distinguished from the non-malignant counterpart by the expression of tumour-associated antigens. Such antigens are often present in high concentrations on tumour cells but sparsely expressed on normal cells [30]. CO17-1A is a common tumour-associated antigen on CRC [16]. Mouse monoclonal antibodies (mAb) (IgG2A) for therapeutic use have been raised against this antigen. Objective responses and even complete remissions have been obtained [4, 26, 27, 34, 38].

The in vivo mechanisms of tumour cell destruction by mAb are not completely understood. In animal and in vitro experimental systems, antibody-dependent cellular cytotoxicity (ADCC) has been claimed to be one of the effector functions [15]. ADCC is mediated by various cells (monocytes; natural killer, NK, cells; granulocytes) through binding of the antibody to Fc IgG receptors (FcR) [8]. Three different classes of FcR have been identified (FcRI, FcRII and FcRIII) [10, 22, 31, 33, 37].

If ADCC is an important effector mechanism, the therapeutic efficacy of mAb might be increased by augmentation of the lytic capability in vivo. The effect is dependent on the total number of killer cells, the number of FcR per cell, the affinity of FcR and the "state of activation" of the cytotoxic cells.

Granulocyte/macrophage-colony-stimulating factor (GM-CSF) is a cytokine that regulates production and differentiation of granulocytes and monocytes/macrophages [29]. GM-CSF influences various effector functions such as inhibition of neutrophil migration [12], stimulation of neutrophil phagocytosis [9], enhancement of macrophage and granulocyte spontaneous cytotoxicity as well as antibody-mediated tumoricidal activity in vitro [7, 13, 24, 25]. We have recently shown that human peripheral blood mononuclear cells (PBMC; lymphocytes and monocytes), preactivated with human recombinant GM-CSF in vitro, significantly increased the lytic capability in ADCC using a human CRC cell line and mouse mAb 17-1A [24, 25]. The mechanisms by which GM-CSF induce these pleiotropic effects are not completely understood.

On the basis of our therapeutic experience with mAb 17-1A alone [11, 28, 35], and on our in vitro results with

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GM-CSF and mAb 17-1A, a treatment protocol for patients with metastatic CRC was designed combining GM-CSF and mAb 17-1A with the aim to augment the lytic function of cytotoxic cells and thereby increase the therapeutic potential of mAb 17-1A. In the present report we describe in vivo changes of cytotoxic functions of blood mononuclear cells that might influence the therapeutic effect of mAb. Some of the therapeutic results have recently been published [28] and a complete report will follow later.

Materials and methods

Patients. Eighteen patients entered a phase IB/II trial; 12 were male and 6 female. The median age was 64 years (range 14–78 years). All patients had advanced metastatic CRC and a Karnowsky index of at least 80%. The tumours expressed the antigen CO17-1A [35]. One patient had received chemotherapy and irradiation 5 months earlier and another chemotherapy 2 months before the administration of GM-CSF and mAb 17-1A. All the others were, except for primary surgery, untreated. The median time from primary surgery to therapy was 4 months (range 1–115 months).

Control donors. A group of 27 healthy individuals was used as control donors. The median age was 39 years (range 24–62 years). None was on medication or had any infectious disease.

Treatment schedule. Recombinant human GM-CSF was produced in *Escherichia coli* (Behringwerke AG, Marburg, FRG). The specific activity was 5×10^7 U/μg protein, and 250 μg/m^2 was given subcutaneously (s.c.) daily for 10 days. The injection was administered at the same time each day. On day 3, 400 mg mAb 17-1A (mouse IgG-2A; Centocor, Malvern, Pa., USA) was administered as an intravenous infusion for 60 min. The treatment cycle was repeated every 4th week. Four cycles were given.

Before each treatment mAb 17-1A (1.0 mg) was injected intradermally, and the skin reactivity was read after 20 min. In patients showing a positive skin reaction the dose schedule of mAb 17-1A was changed. Small doses of mAb 17-1A (1–2 mg) were given as daily infusions over a period of 1–4 h with the aim of preventing an immediate-type allergic reaction. Such a reaction may be caused by antibodies but also by complement activation, histamine and cytokine release [5]; it may be circumvented by a prolonged and slow infusion rate and by administration of low doses of mAb. At the time of this report the patients had received various numbers of treatment cycles and doses of mAb. All patients received a full dose of mAb during the first cycle. At the second cycle, 14 patients received 400 mg mAb while 3 patients were given between 5 mg and 65 mg mAb 17-1A. At the third cycle, 5 patients received a full dose and 10 patients 5–65 mg mAb. Nine patients completed four cycles; only 1 of them received a full dose of mAb 17-1A while 8 patients received 5–65 mg mAb.

Isolation of peripheral blood mononuclear cells (PBMC). PBMC (lymphocytes and monocytes) were obtained by centrifugation of heparinized venous blood on a Ficoll/Isopaque (Pharmacia, Uppsala, Sweden) gradient (density = 1.077 g/ml) and washed three times in Hank's/TRIS solution [2].

Mononuclear cell subsets. Mononuclear cells were analysed for subpopulations by indirect immunofluorescence using absorbed fluorosceinated-isothiocyanate-conjugated goat anti-(mouse IgG) antibodies or rabbit anti-(mouse IgG) F(ab')$_2$ fragments in the second step (B-D, Mountain View, Calif., USA; Dako A/S, Copenhagen, Denmark). Before staining, the cells were incubated at 37° C for 30 min and washed twice in Hank's/TRIS solution. The method has been described in detail elsewhere [21]. The following antibodies were used: Leu-M3 (CD14, 70%–90% of blood monocytes, no reactivity with lymphocytes), Leu-19 (CD56, NK cells, T cells) (B-D), 3G8 (CD16, FcRIII, macrophages, NK cells and some granulocytes), 32.2 (CD64, FcRI, monocytes, macrophages), and IV.3 (CD32, FcRII, neutrophils, monocytes, macrophages, eosinophils and B cells). A sample of 10^4 cells was analysed by flow cytometry (FACScan, B-D) at 480 nm with a flow rate below 300 cells/s. Fluorescence was analysed from scattered/gated viable lymphocytes and monocytes.

Cell line. The target cells used were SW948, a human colorectal cancer cell line, expressing the antigen CO17-1A [14].

Cytotoxicity tests. The cytotoxic activity was determined in an 18-h ^{51}Cr-release assay. After trypsinization of the target cells and testing for viability (trypan blue) the cells were labelled with 2.8 MBq sodium [^{51}Cr]chromate/ml cells (sp. act. = 9–18.5 GBq/mg chromium; Amersham International, UK) at 37° C for 1 h. After washing three times in full medium (Leibovitz medium 15, Gibco Ltd., Scotland, containing 10% fetal calf serum, antibiotics and 2 mmol L-glutamine) the cells were added to round-bottom wells of a 96-well microtiter plate (Nunc, Roskilde, Denmark). The effector cells were preincubated at 37° C for 30 min and washed three times to remove mouse IgG absorbed in vivo and then added to give effector-to-target cell ratios of 50, 25, 12.5 and 6.25. mAb 17-1A was added at a concentration of 10 μg/ml. Supernatants were harvested by the Skatron Titer Tec System (Skatron A/S, Lierbyen, Norway) and counted in a gamma counter. Maximum isotope release was determined by incubation of the target cells with 5% Triton-X (Merck, Darmstadt, FRG). Spontaneous release was determined by incubation of ^{51}Cr-loaded target cells with medium alone. The percentage specific lysis was calculated by the formula

$$\text{lysis (\%)} = \frac{\text{release in sample} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

Results are expressed as lytic units (LU)/10^6 effector cells, where 1 LU was defined as the number of PBMC required to obtain 30% specific lysis according to the method of Pross et al. [14].

Target cells exhibit a dynamic variation in susceptibility to lysis depending on the day after subculturing (unpublished data). To minimize and counterbalance these variations as well as the interindividual variations, a healthy control was included in each experiment. All treatment cycles started on the same day of the week. The subcloning of the cell line (SW948) was also done on the same day. An index was calculated based on the cytotoxicity (LU) of the patient's cells divided by the mean cytotoxicity of cells from the control donors for that day. The mean cytotoxicity (LU) of controls was based on results from 27 individuals except in Figs. 3–5 and 8 (see figure legends). The lytic index (LI) is expressed both as a relative and an absolute value. The relative LI is the lytic capability of 10^6 effector cells. However, the lytic capacity in vivo should be related to the total number of available effector cells. During treatment the total numbers of PBMC varied considerably. This has to be taken into consideration when estimating the lytic capacity of a patient. Thus, an absolute LI was calculated on the basis of the total number of PBMC.

Statistical analyses. Analyses of differences between groups were determined by Student's *t*-test or the Wilcoxon signed-rank test for paired and unpaired observations. As the total numbers of white blood cells are logarithmically distributed, the values were logarithmically transformed before analyses. Statistical analyses were done using Medlog (Information Analysis., Mountain View, Calif., USA) and Instat (GraphPAD Software, San Diego, Calif., USA) software programs.

Results

During treatment with GM-CSF there was a biphasic rise in the total number of white blood cells (Fig. 1 a). The same pattern was noted for neutrophils (Fig. 1 b), eosinophils (data not shown), lymphocytes (Fig. 1 c) and monocytes (Fig. 1 d). The highest value of each cycle was statistically

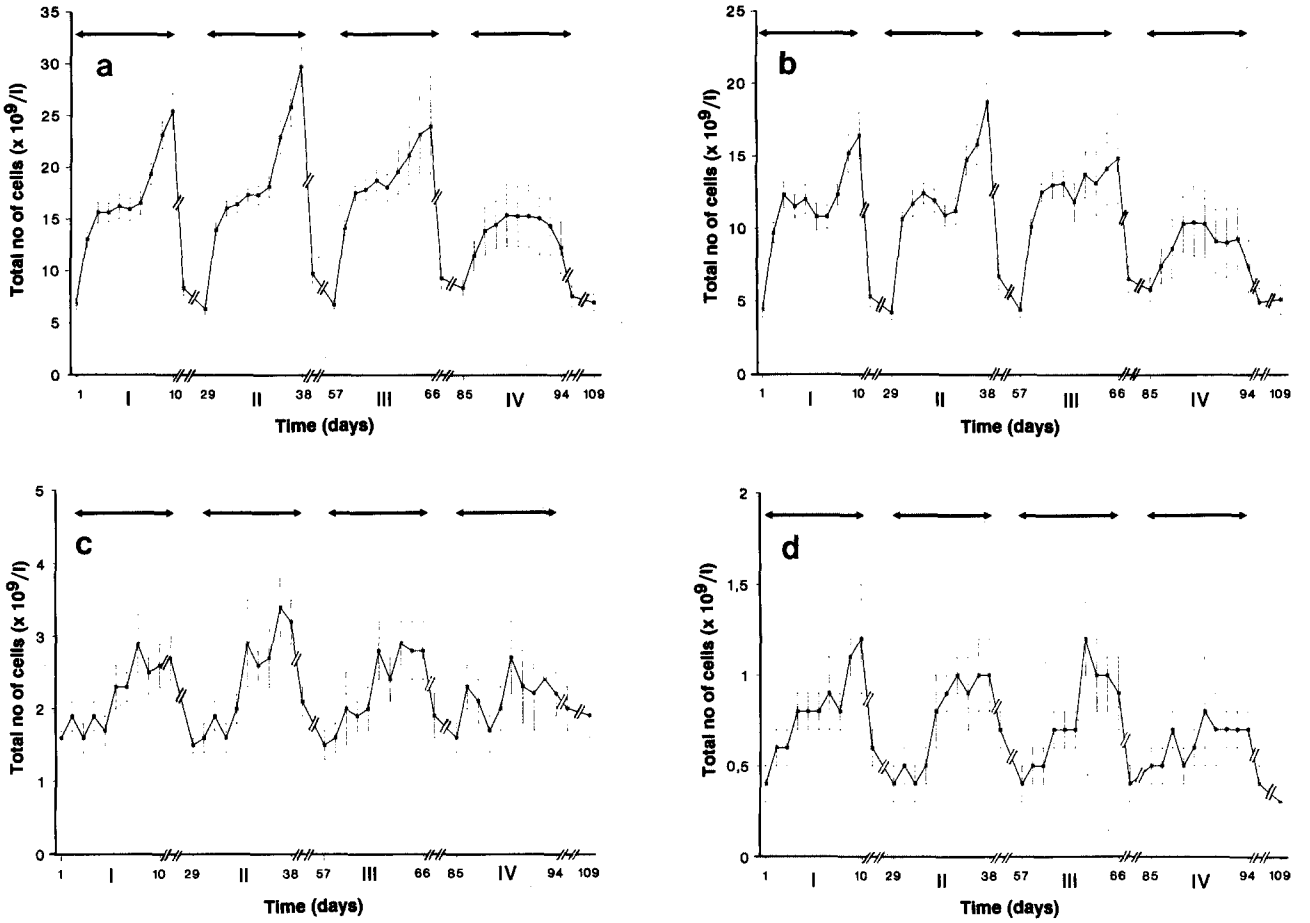

Fig. 1. Total numbers (mean ± SEM) of white blood cells (**a**), neutrophils (**b**), lymphocytes (**c**) and monocytes (**d**) during the four treatment cycles of granulocyte/macrophage-colony-stimulating factor (GM-CSF) (◄—►). *I–IV*, treatment cycle number

Table 1. Frequency (mean ± SEM) of white blood cells before (day 1) and after 10 days of granulocyte/macrophage-colony-stimulating factor (GM-CSF) treatment[a]

Cell subset	White blood cells (%)		*P*
	Day 1	Day 10	
Lymphocytes	23.8±1.6	14.5±1.5	<0.001
Monocytes	6.1±0.4	4.5±0.4	0.01
Eosinophils	4.5±0.6	17.8±1.1	<0.001
Neutrophils	65.1±1.8	60.9±1.5	NS
Basophils	0.5±0.1	0.1±0.1	0.01

[a] The data are based on the values of the first three cycles. NS, not significant

significantly increased compared to the initial value (P <0.01). The rise during the first three cycles was 4.5- to 5.5-fold. At the fourth cycle, the increase in white blood cells was less marked compared to the first three cycles. The peak value of the fourth cycle was significantly lower than the peak values of the three previous cycles (P <0.05). The peak value of the fourth cycle was noted on day 7, while in the preceding courses the highest values were observed on day 10. The relative frequency of eosinophils increased significantly while that of lymphocytes, basophils and monocytes decreased. The proportion of neutrophils was unchanged (Table 1). The cell numbers returned to baseline levels within 5 days after discontinuation of therapy.

Active mononuclear cells in ADCC are monocytes and NK cells. These cells bind the mAb through their FcR. The absolute number of cells expressing FcRI (CD64), FcRII (CD32) and FcRIII (CD16) increased significantly during the first three GM-CSF treatment cycles (Table 2). $CD14^+$ cells (monocytes) also increased. At the fourth cycle, no statistically significant difference in these subsets between days 1 and 10 was noted (data not shown). The absolute numbers of NK cells ($CD56^+$) did not change significantly during either cycle but the relative frequency decreased (Table 2). The variations in these subsets during a treatment cycle is shown in Fig. 2. $CD56^+$ cells remained constant. $CD16^+$ and $CD64^+$ cells increased significantly (P <0.05) and showed a marked peak value at day 6. $CD32^+$ cells increased continuously (P <0.05).

ADCC of PBMC was analysed at diagnosis in all patients. The absolute cytotoxicity (LU) for the patient group was 11.74±2.47 as compared to 6.21±0.96 LU for controls (P <0.05). The corresponding figures for relative ADCC (LU/10^6 cells) were 6.36±1.66 and 3.23±0.5 respectively (P = 0.06).

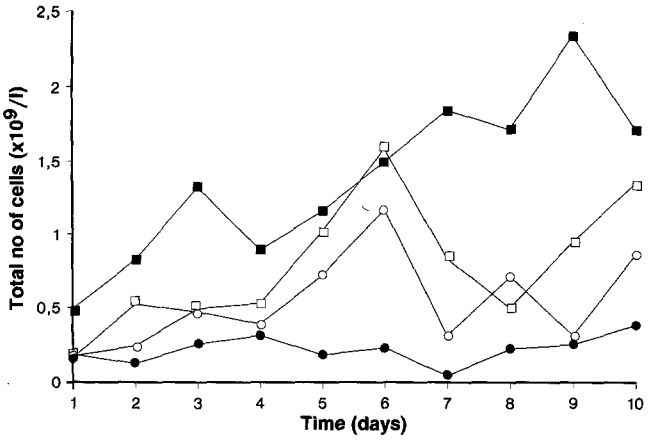

Fig. 2. Total number (median) of CD16+ (○——○), CD32+ (■——■), CD56+ (●——●) and CD64+ (□——□) cells during a 10-day GM-CSF treatment period ($n = 8$). (The tests were done during the second cycle)

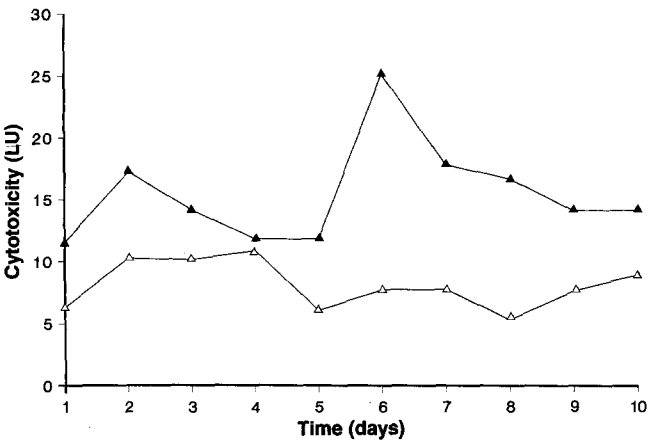

Fig. 3. Absolute antibody-dependent cellular cytotoxicity (ADCC) activity (median) (LU/10^9 cells) of patients ($n = 8$) (▲——▲) during a 10-day GM-CSF treatment period, and the cytotoxicity of concomitant control donors (△——△) ($n = 8$). (The tests were done during the second cycle)

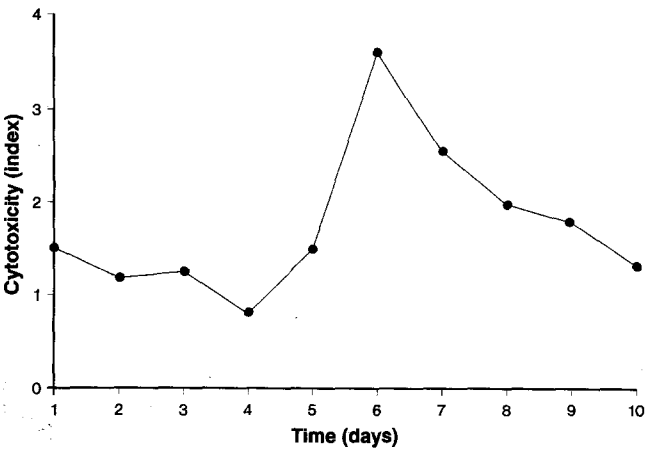

Fig. 4. Absolute ADCC activity (index; median) of patients ($n = 8$) during a 10-day GM-CSF treatment period. The activity on day 6 was significantly increased compared to day 1 ($P < 0.05$)

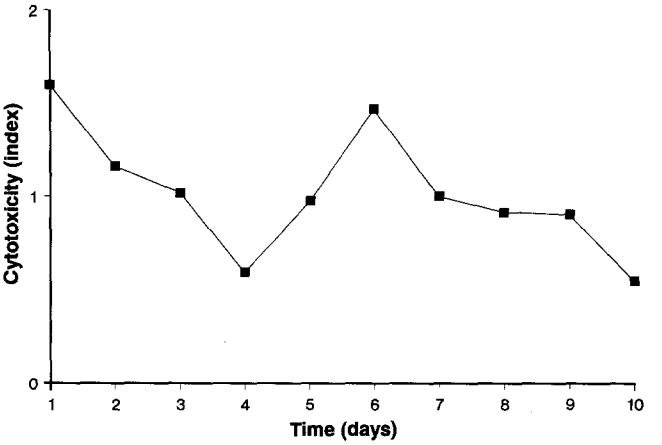

Fig. 5. Relative ADCC activity (index; median) of patients during a 10-day GM-CSF treatment period (compare Fig. 4)

In 8 patients the ADCC capacity of PBMC was studied daily during 10 days of GM-CSF administration. All the patients received 400 mg mAb 17-1A. The absolute ADCC activity of patients in comparison to control donors is shown in Fig. 3. However, chromium-labelled tumour target cells may show a varying degree of susceptibility to lysis depending on the day after subculturing, as demonstrated in Fig. 3. When presenting cytotoxicity data it is important to relate the activity to that of control donors to minimize the day-to-day variations and the interindividual variations. Thus, all cytotoxicity data below are presented as indices. The ADCC activity during the 10 days of GM-CSF treatment (the same as depicted in Fig. 3), presented as indices, is shown in Fig. 4. ADCC was augmented on days 6–9 compared to day 1 although the difference was statistically significant only on day 6 ($P < 0.05$). The relative ADCC also showed a peak value on day 6 but the overall activity declined with time (Fig. 5). The values on

Table 2. Relative and absolute numbers of various cell subsets (mean ± SEM) on days 1 and 10 of GM-CSF treatment[a]

Cell subset	Relative numbers (%)		P	$10^{-9}\times$ Absolute numbers (l^{-1})		P
	Day 1	Day 10		Day 1	Day 10	
CD16	12.4±0.8	28.8±2.6	<0.001	0.23±0.02	1.16±0.15	<0.001
CD32	21.8±1.9	43.6±4.4	<0.001	0.41±0.04	2.09±0.21	<0.001
CD64	9.32±1.7	12.0±2.2	NS	0.17±0.04	0.54±0.10	<0.001
CD14	15.3±1.4	15.2±1.9	NS	0.29±0.03	0.58±0.09	0.002
CD56	10.9±1.2	6.4±1.1	0.006	0.21±0.02	0.25±0.05	NS

[a] The data are based on the values of the first three cycles. NS, not significant

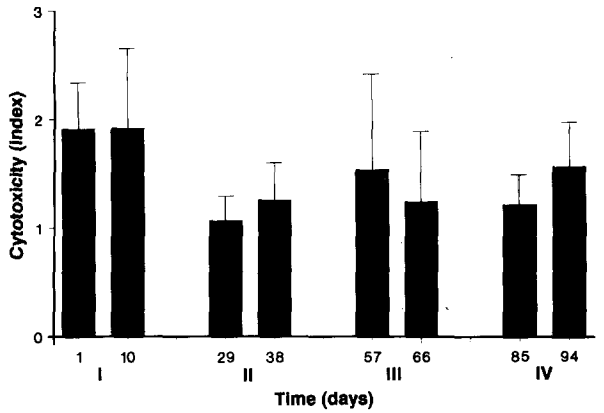

Fig. 6. Absolute ADCC (index; mean ± SEM) of patients treated with GM-CSF for 10 days on days 1 and 10 of the four treatment cycles. *I–IV*, Treatment cycle number

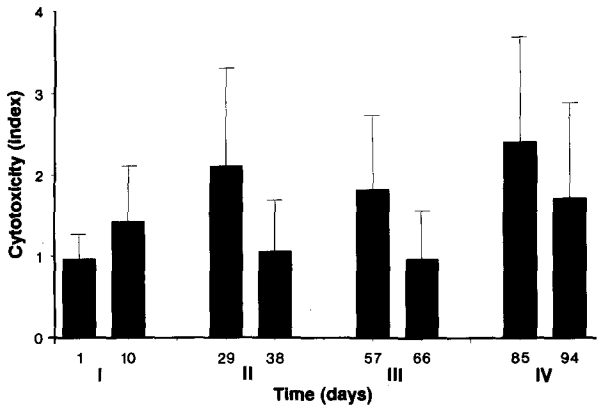

Fig. 7. Absolute spontaneous cytotoxicity (index; mean ± SEM) of patients treated with GM-CSF for 10 days on days 1 and 10 of the four treatment cycles. *I–IV*, Treatment cycle number

days 4 and 10 were statistically significantly lower than on day 1 ($P < 0.01$). Absolute ADCC values of all patients on days 1 and 10 of all the GM-CSF treatment cycles are shown in Fig. 6. No statistically significant difference was found between results on these days or between the various cycles.

At initiation of therapy, the spontaneous absolute cytotoxic activity of PBMC against the NK-resistant cell line SW948 was of the same order of magnitude in the patient as in the control group, 0.91 ± 0.28 LU and 0.81 ± 0.61 LU respectively. The lytic capacity on days 1 and 10 of all treatment cycles was not statistically significantly different, although there was a great individual variation (Fig. 7). During a GM-CSF treatment cycle an increased spontaneous cytotoxicity was found on days 6–8 (Fig. 8), similar to the pattern noticed for ADCC (compare Fig. 4).

Owing to side-effects against mAb 17-1A, the majority of patients received low doses of this mAb during the third and fourth treatment cycles (see Materials and methods). However, there was no significant difference between cycles where a high or a low dose of mAb 17-1A had been given with regard to cytotoxic activity and cell subsets (data not shown).

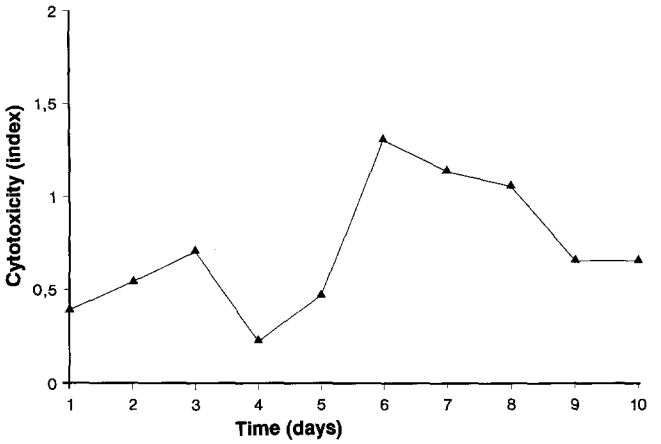

Fig. 8. Absolute spontaneous cytotoxicity (index; median) of patients during a 10-day GM-CSF treatment cycle (compare Fig. 4)

Discussion

Unconjugated mouse mAbs alone have antitumour effects in humans. One of the effector functions seems to be ADCC. As shown in experimental systems [3, 23, 24, 39], the therapeutic efficacy in humans may be enhanced by activating effector cells participating in ADCC. Cytokines may be suitable candidates to achieve this goal. In our therapeutic strategy for the treatment of colorectal carcinoma (CRC) with mAb 17-1A, we have combined the mAb with GM-CSF. The reason for selecting GM-CSF was the enhanced killing of tumour cells in vitro using monocytes and lymphocytes in the presence of mAb 17-1A [24], the simplicity of administering GM-CSF (s.c. once a day) and the favourable profile of side-effects. After therapy with mAb 17-1A monocytes/macrophages increased in the tumour lesion and were localized around the tumour glands. NK cells also increased [35].

Four cycles of GM-CSF were given to the patients. Peripheral polynuclear cells increased as well as monocytes but the lymphocytes increased also. The increase was biphasic but less pronounced at the fourth cycle. Before the initiation of therapy ADCC activity of PBMC was augmented in patients compared to healthy donors. During the 10-day treatment period, blood ADCC was increased on days 6–9, although a statistically significant augmentation was noted only on day 6. ADCC had returned to the pretreatment level by day 10. Spontaneous cytotoxicity against an NK-resistant (CRC) cell line also showed the same pattern of activity. Cells of importance for ADCC ($CD14^+$, $CD16^+$, $CD32^+$, $CD64^+$) increased significantly during the 10-day treatment period.

The biphasic increase in white blood cells during continuous GM-CSF administration has been observed previously [18, 20]. This pattern was only noted in patients receiving more than 3 $\mu g\ kg^{-1}\ day^{-1}$ GM-CSF. As in the present study, the first peak was registered 2–3 days after onset of therapy and a second rise in circulating leucocytes started on days 6–8 of treatment. It is probable that the first phase represents release and more rapid maturation of committed precursors and that the second phase is due to new cell production. The appearance of immature circulat-

ing forms supported this supposition. A decrease in neutrophils on day 4 has also been noted previously [20]. In the present study there was a simultaneous decrease in cytotoxic activity. This decrease might reflect variations in cell subsets participating in ADCC due to the GM-CSF administration. However, the possibility can not be excluded that the infusion of mAb 17-1A on day 3 contributed to the reduced levels of active cells in blood. Tentatively, potential cytotoxic cells may be directed to the tumour by the mAb. The reduced cytotoxic capacity was probably not due to an inhibitory effect of mouse IgG bound in vivo, as PBMC, collected on day 4 and preincubated for 45 min at 37° C (to remove absorbed IgG), exhibited the same cytotoxic potential as non-preincubated cells (data not shown). Similar to our results, serum neopterin levels were sometimes found to decrease shortly after an infusion of an anti-idiotypic mAb to a chronic lymphocytic leukaemia (B-CLL) patient, and this was interpreted as indicating reduced levels of active monocytes, but subsequently the levels rose as an indication of monocyte activation [1].

During the fourth GM-CSF cycle the rise in leucocytes and mononuclear cell subsets was not as marked as during the first three cycles. Administration of GM-CSF s.c. repeatedly for 10 days has not been reported previously. In a study by Lieschke et al. [20] there was a second 10-day retreatment period resulting in the same rise as during the first course, similar to our results. Also, during the third course in the present study the rise was the same. The reason for the low rise at the fourth cycle is not yet clearly understood. Maybe anti-GM-CSF antibodies hampered the effect of GM-CSF. Anti-GM-CSF antibodies were detected in practically all patients at the end of the fourth cycle (to be published).

The increase in lymphocytes was consistently noticed among our patients. Previously lymphocytes have only occasionally been reported to increase at GM-CSF administration [18, 20]. This might be explained by the fact that most patients receiving GM-CSF in other studies have been heavily pretreated with chemotherapy and/or irradiation or immunocompromised by the disease.

GM-CSF has been shown to augment the cytotoxic activity of mononuclear cells slightly (lymphocytes and monocytes) and to increase the ADCC ability markedly in vitro [24]. GM-CSF also increased the expression of FcRII [19], which receptor is of significance for monocyte ADCC as well FcRIII and FcRI on NK cells and macrophages. FcRI binds monomeric mouse IgG2A with high affinity, while FcRII and FcRIII bind polymeric IgG [8].

The spontaneous cytotoxicity of PBMC showed a modest transient increase during the administration of GM-CSF. Kleinerman et al. [18] observed no activation of monocyte cytotoxicity after GM-CSF administration nor could Steis et al. [36] notice increased production of H_2O_2 (measurement of monocyte activation) after stimulation with phorbol myristate acetate. The augmented spontaneous cytotoxicity noticed in the present study is thus probably due to activation of lymphocyte subsets.

The ADCC capability is determined by several factors: the cell phenotype, the expression of FcR, the state of activation and/or the ontogenic state of the cell. In normal blood, 60% of fresh monocytes mediate ADCC. The functional diversity is suggested not to depend on discrete populations of cells but on a continuous maturational/activational state [3]. Thus, during the first days, readily available mature cells in the blood may be activated to give increased ADCC activity. However, during further treatment, cytotoxicity decreased in spite of the fact that cells exhibiting molecules of importance for ADCC continued to increase. This imbalance might be explained by a different activational/maturational stage of the effector cells on day 10 compared to that of the previous days of therapy. The discrepancy between the expression of surface molecules and the lytic capacity might be due to the presence of immature cells [20] not expressing the full lytic machinery. The finding of our study seems to be somewhat contradictory to that of Wing et al. [40] showing that a 10-day administration of GM-CSF only increased monocyte ADCC. However, they used purified monocytes and a different assay (xenogeneic target cells; chicken red blood cells) and ADCC is influenced by the type of target cells [8].

To reach optimal ADCC activity in blood, 6 days were required but then the activity gradually declined, probably as a result of release of immature cells into the blood. This does not necessarily mean that the ADCC activity in the tumour decreased. In vitro GM-CSF has been shown to differentiate monocytes to macrophages and thereby increase the ADCC activity as well as the spontaneous cytotoxicity. The effect was optimal after 8–12 days of culture [41]. Furthermore, tissue macrophages arise from monocytes, which mature once they have left the circulation [17], and the passage over the vessel walls might be facilitated by GM-CSF [6]. Tissue macrophages were also tremendously increased in the tumour lesion during therapy (to be published).

Combinations of mAb and cytokines in the therapy of cancer patients need to be tested in the clinic as these have shown promising results in experimental systems. The present treatment regimen has induced to long-lasting complete remissions in patients with advanced colorectal carcinoma (to be published).

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