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Functional assessment in vitro of human-complement-dependent antibody-induced cytotoxicity of neoplastic cells

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Abstract The complement system is one potential cytotoxic effector mechanism that might be effective in immunotherapy of cancer using monoclonal antibodies (mAb) directed against tumor antigens. In order to evaluate the treatment outcome from trials using mAb in cancer patients, assessment of complement-dependent cytotoxicity (CDC) may therefore be of interest. Here we describe the elaboration of a CDC assay in vitro using a rat hepatoma cell line, H4-II-E, as target cells sensitised with mAb F12, directed against the tumor-associated ganglioside antigen fucosyl-GM1. Sensitised cells were incubated with various concentrations of fresh serum as complement source for 48 h and cytotoxicity was then assessed by the tetrazolium bromide (MTT) test. A large variation in CDC efficacy was observed between individual serum donors. No differences in CDC could be seen between healthy donors and cancer patients. The CDC showed a strong correlation to the serum concentrations of complement factor C4, supporting the validity of the assay. Our results suggest that there may be significant variations in complement function within and between individuals that might influence the outcome of clinical mAb therapy. The H4/F12 CDC assay described here, together with measurement of individual complement factors, such as C4, should be further validated in cancer patients at various disease stages and phases of treatment.

Key words Monoclonal antibody · Complement-dependent cytotoxicity · Ganglioside fucosyl-GM1 · In vitro assay

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Introduction

It has been estimated that approximately 50% of cancer patients can be cured after uni- or multimodality treatment involving surgery, chemotherapy and radiotherapy. For the remaining patients additional treatment modalities, such as immunotherapy with monoclonal antibodies (mAb) directed against tumor-associated antigens, might result in an improved prognosis. Up to now a large number of potential tumor-associated target antigens on tumor cells, such as lung cancer [3, 22, 29], colorectal cancer [1, 6], malignant melanoma [26, 30], and neuroblastoma [5], have been identified. mAb bound to tumor cells can activate the complement system with deposition of complement factor complexes that may result in tumor cell death (complement-dependent cytotoxicity, CDC) [19]. Other mechanisms through which mAb can induce tumor cell growth retardation and/or death include induction of apoptosis [28], inhibition cell membrane signal transduction from mitogens [20, 34] and activation of lymphoid cells for antibody-dependent cellular cytotoxicity [12, 31]. Administration of mAb to patients with neuroblastoma has been shown to activate the complement system and also possibly result in longer survival [5].

mAb-induced complement activation leading to cell death is a concerted and complicated series of reactions involving complement factors C1 through C9 and other regulatory proteins. To assess complement cytolytic activity, the CH50 assay, using sensitized sheep red blood cells as targets, is commonly used [8, 15]. Since red blood cells may be more sensitive than neoplastic cells to the cytolytic action of complement, the CH50 assay may not be adequate for assessment of CDC activity against tumor cells in cancer patients. Since complement function may be important for the effects of mAb therapy, an in vitro assay using neoplastic target cells should probably be used. No such assay has yet been described.

Here we describe the elaboration and initial evaluation of an in vitro assay consisting of the rat hepatoma

cell line H4-II-E expressing a fucosyl-GM1 ganglioside [2, 3] and a sensitising murine mAb F12 (IgG3) that reacts with fucosyl-GM1 ganglioside [9] for the assessment of functional CDC activity in human serum. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test was used to measure the fraction of surviving cells [21]. The results were compared with the serum concentrations of two complement factors, C3 and C4.

Materials and methods

H4-II-E cells and mAb F12

The carcinogen-induced rat hepatoma cell line H4-II-E [24] was purchased from ATCC (Rockville, Md.). This cell line has a high and homogeneous expression of the tumor-associated ganglioside antigen fucosyl-GM1 [2, 3]. The cells grow as a monolayer and were maintained in Iscove's medium supplemented with 4 mM L-glutamine, 100 μg/ml streptomycin 100 U/ml penicillin G and 10% fetal bovine serum (FBS) in 25-cm^2 plastic flasks at 37 °C in an atmosphere containing 5% CO_2 and with more than 90% humidity (culture conditions). Cells were harvested for passage or for use in tests by treatment with 0.02% EDTA (w/v) in phosphate-buffered saline (PBS; 0.01 M phosphate buffer, pH 7.4, with 0.14 M NaCl) for 5 min under culture conditions, and then washed in sterile PBS. Cell viability, as assessed by trypan-blue dye exclusion, was above 90% for the cytotoxicity tests. The production of the murine mAb F12 (IgG3κ) has been described earlier [23]. mAb F12 has a high specificity for the fucosyl-GM1 ganglioside [9]. Antibody in hybridoma culture supernatants was purified by fast protein liquid chromatography on protein-A-Sepharose (Pharmacia). Solutions of 2 mg/ml mAb in PBS were sterile-filtered and stored at −70 °C until use. The mAb was diluted in cell culture medium for use in cytotoxicity tests.

Complement

Human serum was used as source of complement. Peripheral venous blood was drawn from healthy members of the staff ($n = 15$) and from patients ($n = 7$) with clinically manifest cancer of various histotypes before the start of any cancer treatment. Serum samples were also taken from 3 cancer patients at three to five assay times during their first cycle of chemotherapy. The blood was allowed to coagulate at room temperature for 30–60 min and then centrifuged. The serum was aspirated, divided into aliquots and kept at −70 °C until use. As complement reference, serum samples from 4 healthy volunteers were pooled before freezing.

Flow immumocytometry

H4-II-E cells were fixed immediately after harvest with 2% formaldehyde in PBS for 5 min at +4 °C and thereafter washed three times with PBS. Fixed cells were suspended at 10^7 cells/ml in PBS with 0.5% bovine serum albumin (BSA) and transferred to V-bottomed 96-well microtest plates (90 μl/well). Wells were incubated with mAb F12 at 10 μl/well at various concentrations for 20 min. Plates were then centrifuged and the supernatant discarded. Cells were resuspended and incubated with fluorescein-isothiocyanate-conjugated F(ab)2 goat anti-(mouse Ig) (no. 1032-02; Southern Biotechnology, Birmingham, Ala.), diluted 1/100 in PBS, with 0.5% BSA 100 μl/well for 20 min. Cells were then washed twice with PBS by centrifugation and finally fixed with 2% formaldehyde in PBS. Flow-cytometric cell sorting was performed in a FACScan (Beckton-Dickinson).

Enzyme-linked immunosorbent assay (ELISA)

Flat-bottomed 96-well plastic microculture plates were seeded with H4-II-E cells at various densities (0.1×10^4–4×10^4 cells/well) in cell culture medium with various concentrations of FBS. After 2–3 h the cells had adhered firmly to the bottom of each well. All culture medium was then removed and each well quickly washed twice with 200 μl PBS. Cells were fixed by addition of 2% formaldehyde in PBS (100 μl/well) for 30 min at room temperature. This fixation was necessary to prevent loss of glycolipid antigen from cells during the subsequent incubation procedure [2]. After fixation, each well was washed twice for 5 min with 200 μl PBS. Blocking of wells to prevent unspecific binding of mAb or antiserum was done by incubation with 200 μl PBS with 7.5% sucrose and 0.5% BSA for 30 min at 37 °C followed by two washings with PBS. ELISA was performed by stepwise incubations with mAb F12 (various concentrations, see Results) and horseradish-peroxidase-conjugated rabbit anti-(mouse Ig) diluted 1/250 (DAKO P 0260; Dakopatts, Copenhagen, Denmark). Each incubation step was done for 2 h at room temperature, using 50 μl antiserum/well diluted in PBS with 0.5% BSA followed by three washings of 2 min with 200 μl/well PBS. Bound horseradish peroxidase was detected by incubation of each well with 100 μl/well *o*-phenylenediamine (tablets from Dakopatts) in 0.1 M sodium citrate buffer pH 4.4 for 10–20 min at room temperature. The absorbance was read in a microtest-plate spectrophotometer (Titertek Multiscan, Labsystems Ltd.) at 450 nm. All tests were done in triplicate.

CDC and tetrazolium bromide (MTT) test

H4-II-E cells were cultured in 25-cm^2 flasks and harvested at log phase as described above. Cells were seeded in flat-bottomed 96-well microculture plates (100 μl/well) at the desired densities in culture medium with 4% FBS and incubated at culture conditions for 2–3 h to let target cells adhere. mAb F12 (sterile-filtered) diluted in culture medium without FBS (75 μl/well) was added. The complement source (fresh human serum) was diluted with culture medium (without FBS) in V-bottomed 96-well microtest plates kept on ice. Diluted complement (25 μl/well) was transferred to the test plates, which were then incubated for 48 h under the culture conditions. All tests were done in triplicate. Cell growth retardation (by cytostasis and/or cell death) was assessed by quantification of viable cells by the colorimetric MTT assay [4, 13, 21]. H4-II-E cells at various densities were incubated under the culture conditions, with or without mAb and/or complement, for 48 h. All the culture medium was then replaced with 100 μl/well cell culture medium containing 2% FBS. MTT (M5655, Sigma), 5 mg/ml in sterile PBS (10 μl/well), was added and plates were incubated under cell culture conditions for 3 h. The formazan precipitates formed were dissolved by adding 100 μl/well 0.04 M HCl in isopropanol followed by incubation for 60 min at 37 °C. The absorbance was measured in a multiwell plate scanner at 570 nm with 690 nm as the reference wavelength.

The survival index (SI) of each test was calculated, using the following formula:

$$\mathrm{SI}(\%) = 100 \times (T - B)/(M - B)$$

where T is the absorbance of the test sample, B is the mean absorbance of triplicate background wells (only culture medium with FBS) and M is the mean absorbance of triplicate wells with H4-II-E cells in culture medium with FBS but without mAb or complement (maximum absorbance). The mean SI and SD of triplicate tests were then calculated.

Serum C3 and C4 concentrations

Concentrations of C3 and C4 in serum samples were determined in the Behring nephelometer analyzer (Marburg, Germany), using rabbit anti-human C3 and C4 respectively.

Statistical evaluation

Statistical evaluations were carried out, using the StatView software for Macintosh (Abacus Concepts Inc.). Non-parametric comparisons of groups were based on the Mann-Whitney *U*-test. The Spearman rank test was used to evaluate the correlation between serum complement concentrations and survival index in the cytotoxicity assay.

Results

mAb F12 binding to H4-II-E cells

In flow immunocytometry with 10 μg/ml and 2 μg/ml mAb F12, approximately 98% and 87% of the cells were labelled respectively (Fig. 1). In ELISA with H4 cells cultured in medium with 10% FBS, a plateau of mAb F12 binding was seen at concentrations above 6 μg/ml (data not shown). When H4-II-E cells that had been cultured at various concentrations of FBS for 48 h before plating in microtest plates were tested, mAb F12 binding was insignificantly less for H4 cells cultured in 2% than for those cultured in 10% FBS (data not shown). Together these data indicate that optimal binding to H4 cells cultured in 2–10% FBS is achieved using 10 μg/ml mAb F12.

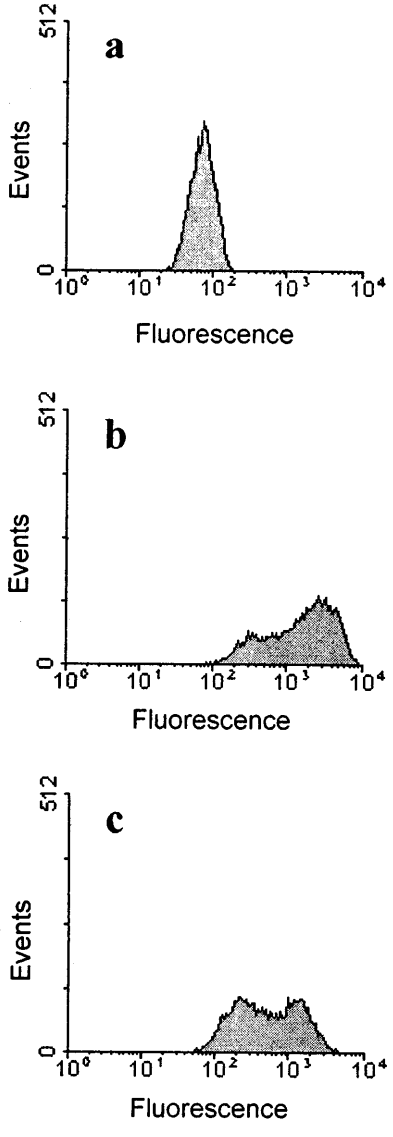

Fig. 1a–c Histograms of flow immunocytometry staining of H4-II-E cells with (**a**) 10 μg/ml mAb O4 (murine IgG3 of irrelevant specificity) (**b**) 10 μg/ml mAb F12, and (**c**) 2 μg/ml mAb F12

MTT linearity of H4-II-E cell density

The linearities of MTT metabolism by H4-II-E cells plated at various cell densities and cultured for 48 h at various concentrations of FBS (1%–10%) are shown in Fig. 2. Good linearity was seen with up to 2×10^4 cells/well in 96-well flat-bottomed microculture plates. No significant differences in MTT metabolism were seen between 2% and 10% FBS. Since high FBS concentrations (above 5%) during MTT metabolism result in high background absorbance in CDC tests, 2% FBS was used subsequently.

CDC test parameters

The following variables in the CDC test were optimized: the seeding density of target cells and the concentrations of mAb and serum samples.

The influence of cell density on the survival index curve was tested at 1×10^4–8×10^4 target cells/well, using mAb F12 at various concentrations and 10% reference human serum. At 2×10^4 target cells/well, effective killing (less than 20% surviving cells) was seen at 10 μg/ml mAb F12. From this and the linear curve of MTT metabolism by H4-II-E cells, a seeding target cell

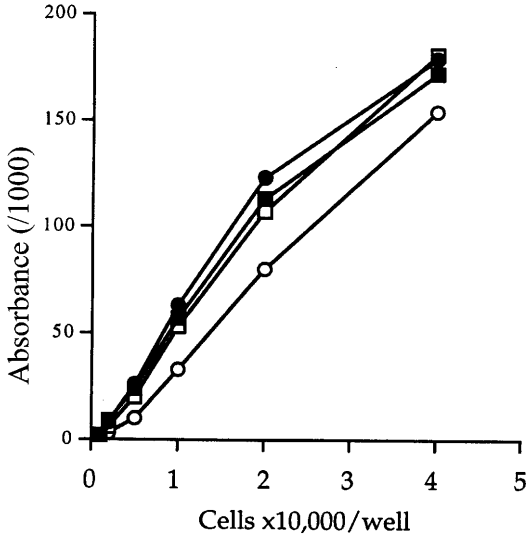

Fig. 2 MTT metabolism of H4-II-E cells cultured for 48 h at various densities. FBS concentrations used during the cell culture and MTT test: ■ 10%, ● 5%, □ 2%, ○ 1%. The mean absorbance ± SD of triplicate tests is shown

density of 2×10^4/well was chosen for the final assay. Titration of mAb F12 together with 10% reference serum showed that 10 μg/ml mAb F12 resulted in approximately 85% cell killing (Fig. 3), whereas 5 μg/ml and 20 μg/ml resulted in approximately 70% and 90% cell killing respectively. On the basis of these results, 10 μg/ml mAb F12 was chosen for the final assay.

Titration of the reference serum showed that the maximum cell killing was achieved at 5–10% serum concentration (data not shown). This cell killing was dependent on the addition of mAb since no killing of cells could be detected at 20% serum without mAb. The use of heat-inactivated serum (56 °C for 40 min) in the presence of mAb F12 also abolished all cytotoxicity. In repeated experiments a considerable interexperimental variability was seen. We therefore tested all serum samples at three different concentrations (5%, 2% and 0.8%). In all experiments the reference serum pool was included. The activities of complement in serum samples were compared at the concentration that, for reference serum, yielded a cell survival as close to 50% as possible in individual experiments. In all experiments this concentration proved to be 0.8% or 2%.

The final settings adopted for the serum CDC assay were as follows: H4-II-E target cells 2×10^4/well, mAb F12 10 μg/ml, and human serum sample (complement source) concentration at the time of testing 5%, 2% and 0.8%.

CDC of test serum samples

Repeated tests of the same serum specimen within the same experiment showed a low intra-experimental variation with a coefficient of variation (CV) of no more than 20% within triplicate tests at 0.5% serum sample concentration. At higher serum concentrations the CV was less. The mean and median survival indexes (SI) for CDC in serum from cancer patients were 35% (±SD 31) and 31% (range 0–89%) respectively, and in the serum from healthy donors they were 46% (±SD 25) and 49% (range 12%–95%) respectively ($P = 0.26$) (Fig. 4a).

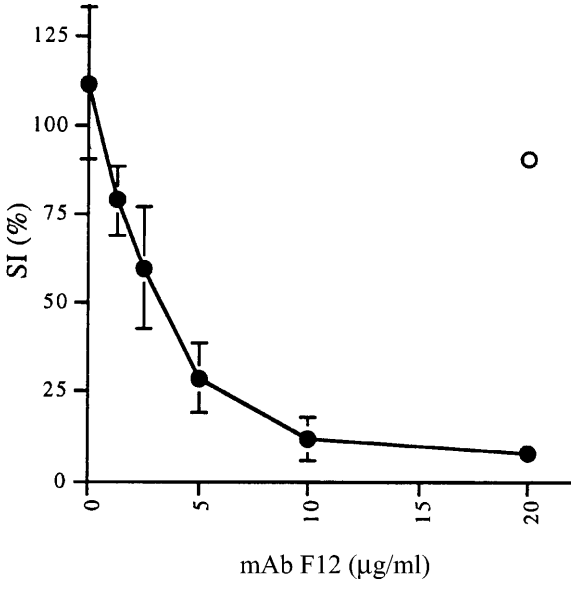

Fig. 3 Complement-dependent cytotoxicity (CDC) test on 2×10^4/well H4-II-E cells with mAb F12 (●) or mAb O4 (○; negative control murine IgG3 mAb with irrelevant specificity) and 10% reference serum. Mean survival index (*SI*) ± SD is depicted

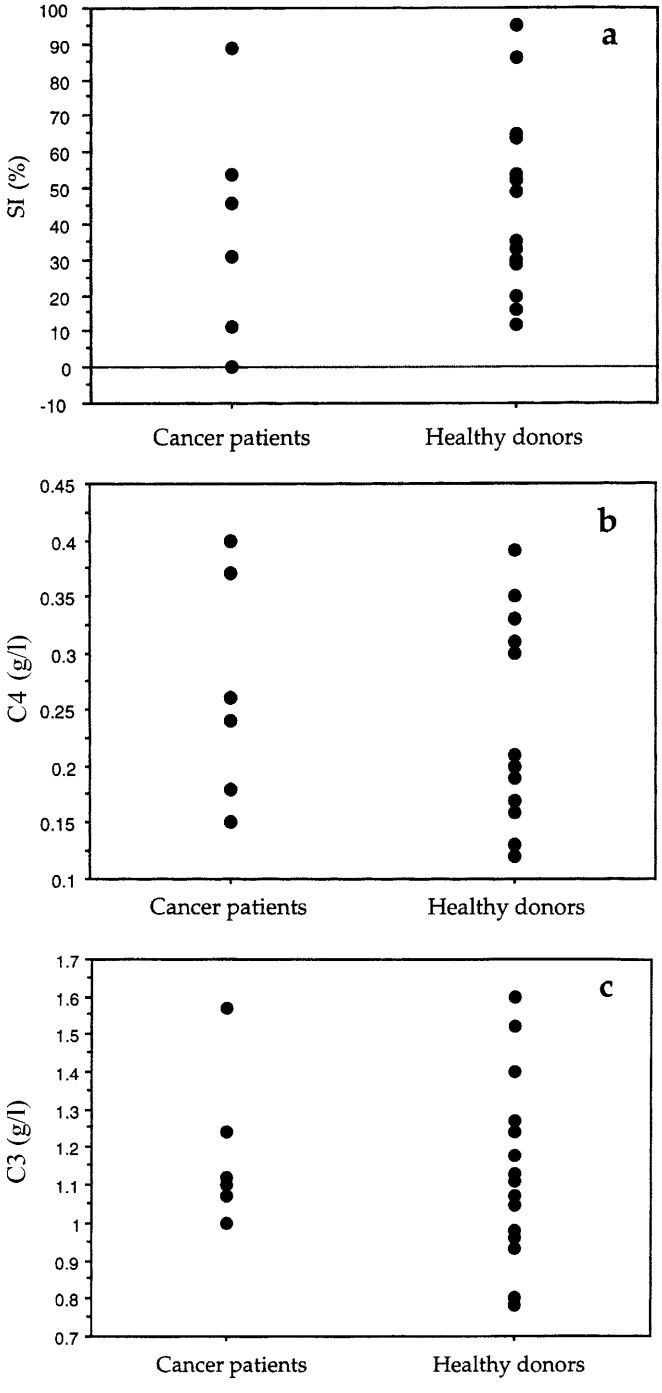

Fig. 4a–c Scattergrams of survival index (*SI*) after CDC (**a**), and concentrations of C4 (**b**) and C3 (**c**) in serum samples from cancer patients ($n = 7$) and healthy donors ($n = 15$). For statistics, please refer to Results

When compared with the reference serum pool, the serum from 4 of 7 cancer patients and 3 of 15 healthy donors showed a higher CDC efficacy.

Serum complement components

The mean and median serum concentrations of factor C4 in cancer patients were 0.26 g/l (SD ± 0.092) and 0.24 g/l (range 0.15–0.40 g/l) respectively, and in healthy donors these were 0.25 g/l (SD ± 0.086) and 0.30 g/l (range 0.12–0.39 g/l) respectively ($P = 0.81$) (Fig. 4b). The corresponding figures for C3 were 1.16 g/l (SD ± 0.20) and 1.10 g/l (range 1.00–1.57 g/l) respectively in cancer patients, and 1.14 g/l (SD ± 0.24) and 1.11 g/l (range 0.78–1.60 g/l) respectively in healthy donors ($P = 0.81$) (Fig. 4c).

There was a slight positive correlation of serum concentrations of C4 and C3, as shown in Fig. 5a ($r_s = 0.32$, $P = 0.14$).

Correlation between CDC and complement components

A significant inverse correlation was seen between the SI of CDC and serum concentrations of factor C4 (Fig. 5b; $r_s = -0.82$, $P < 0.0001$). No significant correlation was seen between C3 and SI (Fig. 5c; $r_s = -0.14$, $P = 0.52$). The results suggest that serum C4 concentrations might be predictive for the cytotoxic function of the complement system in human donors.

Complement during a chemotherapy cycle

Serum samples from three cancer patients (BD, MB and ZC), drawn at various times during their first course of chemotherapy, were tested for CDC and serum concentrations of C4 and C3 (Fig. 6a–c). In all three patients the SI values after CDC at the beginning and the end of the cycle were of the same magnitude. In 1 patient the CDC capacity rose (BD; corresponding to a diminished SI) whereas in another it fell (MB; elevated SI) during the first days of the cycle. In the third patient (ZC) no significant change was seen during the cycle. Dynamic changes could be noted also for the serum concentrations of C4 and C3.

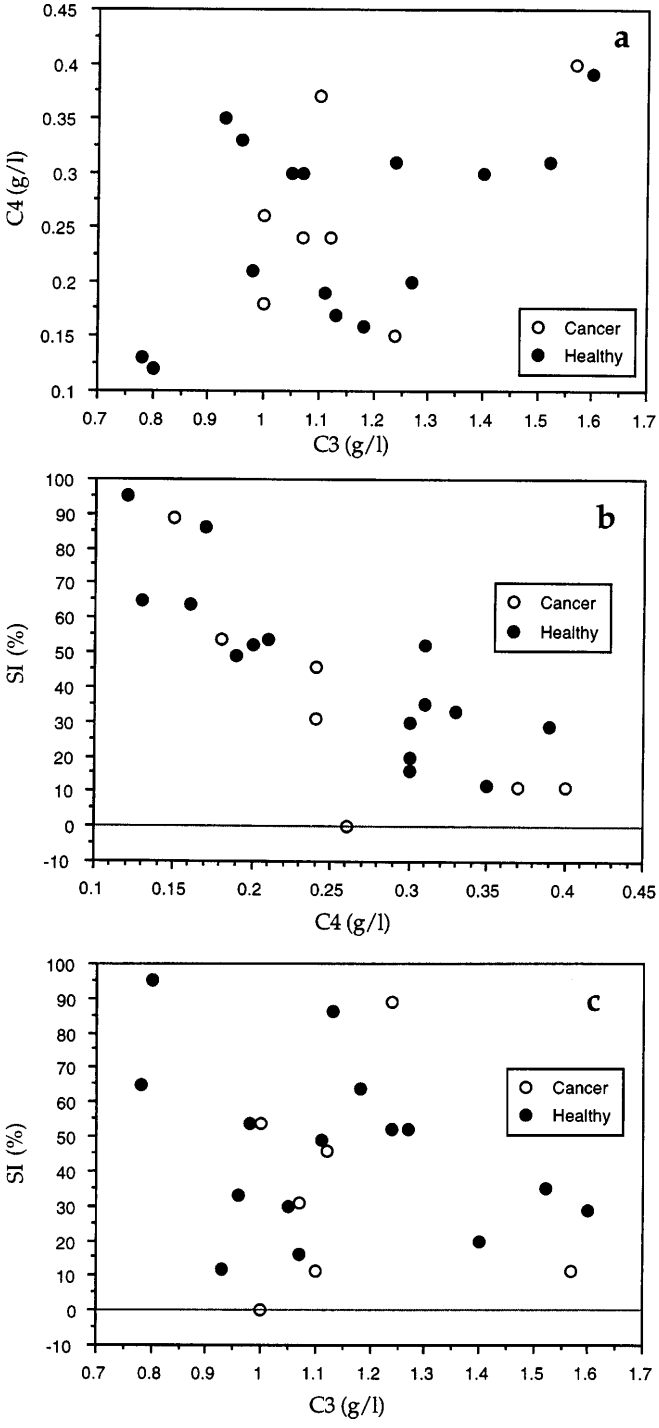

Fig. 5a–c Scattergrams of correlations of serum concentrations of C3 with C4 (**a**), SI with C4 (**b**) and SI with C3 (**c**). For statistics, please refer to Results

Discussion

Complement deposition in tumor lesions and complement activation have been demonstrated in patients treated with mAb for malignant melanoma and neuroblastoma [5, 14], suggesting that complement functions as an effector in passive specific immunotherapy of cancer. At present there is limited knowledge about the function of the complement system in cancer patients at different stages of disease and treatment. In patients with advanced disease and poor nutritional status with weight loss, the production of complement factors may be impaired, resulting in poorer complement function [33]. Chemotherapy might also influence the production and function of individual complement factors. Assays of complement function may become potentially useful

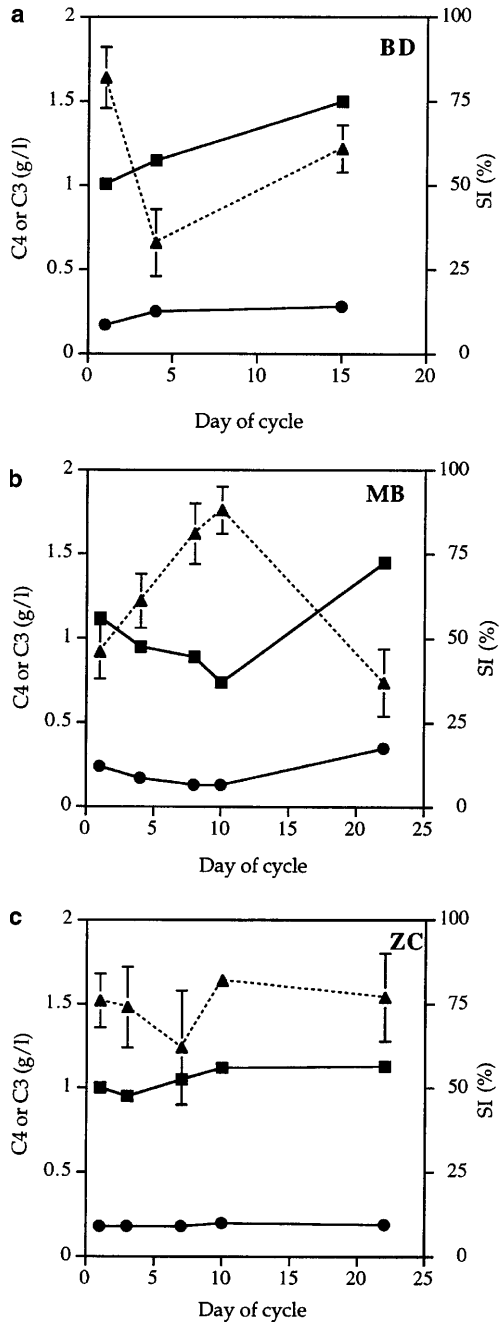

Fig. 6a–c Analysis of serum samples from 3 cancer patients (BD, ZC, MB respectively) at various times during their first chemotherapy cycle. Analyses depicted: concentrations of C3 (■) and C4 (●), and survival index (SI, ▲; mean of triplicate tests ±SD)

to predict the therapeutic outcome of passive immunotherapy using mAb.

Here we have chosen the rat hepatoma cell line H4-II-E (H4) as the target in an assay of mAb-induced CDC. This cell line was regarded as suitable for several reasons. H4 cells are easily grown in standard culture media. After passage of semiconfluent cultures, cells have a short lag phase even at low seeding densities, and the time from seeding to harvest of semiconfluent cultures in log phase with high viability is highly predictable. H4 cells do not activate human complement in serum collected from any of the individuals (healthy staff members or cancer patients) hitherto tested without addition of antibody. This suggests that human serum does not contain natural or endogenous antibodies reacting with H4 cells, and that H4 cells do not activate complement through the alternative pathway that can be seen for certain human neoplastic target cells. H4 cells also appear to be more relevant as targets than erythrocytes for assessing the tumor cytotoxicity of complement, since he latter are more sensitive to complement-mediated cytolysis than nucleated cells [7].

The use of the MTT test is compatible with measurement of the concerted action from several mechanisms of cell death and cytostasis since the formation of formazan is directly proportional to the number of viable cells. The ^{51}Cr-release test, however, which is frequently used to test complement cytotoxicity, is reliable for measuring cytolysis (i.e. lost cell membrane integrity) but probably not for other mechanisms. For CDC using nucleated targets, the MTT test therefore appears highly relevant and was chosen for assessment of the cell viability end-point in the CDC assay. We have subsequently also compared the MTT test with the fluorometric microcytotoxicity assay [16] using fluorescein diacetate as the metabolic substrate in viable cells. This assay, however, produced a high background, mainly because of the contribution from human serum, and therefore proved to be less sensitive than the MTT-based assay (data not shown).

The concentration of mAb F12 (10 μg/ml) used in the assay is clinically achievable and therefore appears relevant. An IgG mAb, instead of IgM, was chosen because passive immunotherapy with unconjugated native antibodies uses the IgG isotype, as it is able to activate both complement and cellular cytotoxicity and should permit an estimate of the mAb-induced cytotoxic capacity of the complement system.

Despite the fact that all known and controllable variables of the CDC assay procedure were kept constant, the interexperimental variability was considerable, making comparisons between experiments difficult. In order to compare the results between different tests within one experiment and also to test a sufficient number of serum samples (15 samples plus reference serum) within one experiment, each test was done in triplicate. This resulted in a coefficient of variation of less than 20% within triplicates, which was regarded as acceptable. For reference, a pool of serum from healthy donors was used. Testing of different batches consisting of serum from 4–6 different healthy donors resulted in identical dose/response curves in the assay (data not shown). Since there was a considerable variability of complement activity in the assay between individual healthy donors, the use of a serum pool instead of individual serum samples as reference seemed appropriate. Serum samples and the reference pool were tested at three concentrations (5%, 2% and 0.8%) in order to ensure that a survival index (SI) of 25%–50% for

the reference pool at any of these concentrations was achieved. Different samples were then compared at a concentration that yielded an SI below but as close to 50% as possible. The validity of this algorithm and of the complement assay is demonstrated by the strong correlation between SI and serum C4 concentrations. This correlation further suggests that C4 is the rate-limiting factor for the classical pathway of complement activation, and that assessment of serum C4 concentrations may be of relevance in patients who are candidates for passive immunotherapy using mAb. Low C4 concentrations, although within the normal reference range, resulting in a low CDC activity might then be correlated to a poor therapeutic response. These hypotheses, however, need to be tested in future studies.

There is a discrepancy among reports on complement factors and their function in cancer patients and healthy individuals. Some describe inferior function [25], whereas others describe increased complement levels [11, 17]. A transient rise in complement factors and function has been observed in cancer patients immediately after surgery [18]. Complement levels may also vary between different types and stages of cancer [10, 27, 32].

Our tests of serum samples from cancer patients who had received chemotherapy show that there is a considerable intra-individual variability in complement cytotoxic function and serum complement concentrations. This may be explained by the influence of cytostatic drugs. Assessment of complement function in patients receiving chemotherapy may therefore be of significance when concomitant immunotherapy with mAb is considered.

The 3 patients studied here illustrate different possible scenarios for complement variability during a chemotherapy cycle. In patient MB there was a fall in C3 and C4, suggesting down-regulation of the complement system possibly resulting from complement activation (consumption) or decreased production of complement factors during the first 2 weeks of the chemotherapy cycle, resulting in decreased CDC activity. In patient BD, there was an increase in C3 and C4, leading to increased CDC. At the end of the chemotherapy cycle, however, there was an increase in concentration of C3, but not C4, accompanied by less CDC activity. In patient ZC no significant changes in C3 or C4 concentrations or in CDC activity were seen. Taken together, these results suggest that the concentration of C4 in serum may be crucial for CDC activity, even if the concentrations of C3 are high. These observations should be extended by further studies.

In summary, we have designed an in vitro assay using neoplastic target cells for assessment of antibody-induced cytotoxic complement function in serum. Using this assay we have found a significant variability in the cytotoxic function of complement activation induced by mAb against neoplastic target cells in healthy individuals and in cancer patients. The complement function, as indicated by the survival index of the assay, showed a strong correlation to the serum C4 concentration, giving support to the validity of the test and further suggesting that C4 may be a relevant marker of CDC activity in the patient. The outcome of the complement assay described, using complement-deficient sera and samples from patients with various inflammatory and neoplastic diseases, should be studied for further validation. Owing to the potential importance of complement as an effector system for mAb-mediated immunotherapy of cancer, the variability of complement function in cancer patients deserves further investigation.

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