

# Anti-Fas/Apo-1 Monoclonal Antibody CH-11 Depletes Glutathione and Kills Multidrug-Resistant Human Leukemic Cells

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**ABSTRACT:** Apoptosis is a common pathway by which cells respond to noxious insults or growth regulatory factors. Since cellular glutathione (GSH) content has long been known to govern response to antineoplastic treatment we have compared induction of apoptosis in drug sensitive (HL-60 and K562/WT) and drug resistant (KG-1a and K562/ADM) human leukemic cell lines by the monoclonal antibody CH-11 (anti-Fas/Apo-1). Fraction of apoptotic cells and cellular GSH were determined by flow cytometry. All cell lines were induced to undergo apoptosis by exposure to mAb CH-11 independent of resistance to conventional antineoplastic treatment. In conjunction with exposure to daunorubicin, vincristine, carboplatin, cytosine arabinoside, dexamethasone, or ionizing irradiation the effect of mAb CH-11 on induction of apoptosis was no more than additive. In contrast, preincubation with IFN-gamma markedly enhanced the induction of apoptosis by mAb CH-11 due to an increase of Fas-receptor expression. In each instance, GSH content decreased with increasing fraction of apoptotic cells indicating a crucial role of GSH in the apoptotic pathway.

**Keywords:** apoptosis, Fas/APO-1, glutathione, drug resistance, leukemia

## INTRODUCTION

It is now commonly accepted that programmed cell death, referred to as apoptosis, plays a major role in maintaining and regulating homeostasis, development, and differentiation of tissues under physiological conditions (1). Apoptosis is also observed in tumors and its relevance to cancer therapy has been emphasized (2). The Fas/Apo-1 antigen has been shown to be an important mediator of apoptosis (3). Cloning of the Fas/Apo-1 coding gene revealed close amino acid sequence homology to the receptors for tumor necrosis factor (TNF) and nerve growth factor (4). It has been demonstrated that monoclonal antibodies against the Fas/Apo-1 protein trigger apoptosis in Fas/Apo-1 expressing cells (5,6).

Although the natural Fas/Apo-1 ligand (presumably mimicked by antibody binding) has been identified (7), the molecular pathways which transduce death signals are not fully understood. Evidence has been provided that TNF cytotoxicity is mediated by free radicals which cause oxidative injury (8). Intracellular glutathione (GSH) provides protection from TNF induced oxidative attack (8,9). It is therefore reasonable to propose that Fas/Apo-1 antibodies may also confer cytotoxic activity by depletion of GSH.

In an attempt to elucidate downstream events of Fas/Apo-1 we analyzed the induction of apoptosis by the anti-Fas antibody CH-11 and its effect on the cellular GSH level in several human leukemic cell lines. GSH has been proposed as relevant mechanism of resistance against different

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cytostatic drugs (10). Therefore, we also addressed the question, whether the antibody CH-11 is capable to affect multidrug-resistant cells.

## MATERIAL AND METHODS

### *Cell Lines*

Cultivation of human leukemic KG-1a, sensitive K562/WT, doxorubicin-resistant K562/ADM, and HL-60 cell lines has been described (11).

### *Treatment of Cells*

Monoclonal antibody (mAb) CH-11 (Kamiya Biomedical Company, Thousand Oaks, CA, USA) against the Fas protein was applied to logarithmically growing leukemic cells in a dose range of 0.01 to 10 µg/ml. Since previous investigations showed that gamma-interferon (IFN-gamma) enhances the sensitivity of cells to anti-Fas mediated cytotoxicity by increasing the expression of Fas receptor molecules (6), aliquots of cells were pretreated with IFN-gamma (100 U/ml; Boehringer Mannheim, FRG) for 24 hrs. Furthermore, the following agents were used: daunorubicin (Daunoblastin, Farmitalia, Freiburg i. B., FRG), vincristine (Lilly, Giessen, FRG), carboplatin (Carboplat, Bristol, Munich, FRG), cytosine-arabioside (Alexan, Mack, Illertissen, FRG), and dexamethasone (Fortecortin, Merck, Darmstadt, FRG). Gamma-irradiation was performed in a <sup>137</sup>Cs source (IBL 437C, Cis Bio International, Gif-sur Yvette, France). All antineoplastic agents were applied in doses which cause only minor growth inhibitory effects (20-30 %). These were taken from previous dose response curves.

### *Determination of Apoptosis*

The appearance of cells less intensively stained than G<sub>1</sub> cells (sub-G<sub>1</sub> or A<sub>0</sub> cells) in flow cytometrical DNA histograms is generally accepted as a marker for apoptosis (12). Reduced

DNA stainability in apoptotic cells is due to DNA fragmentation and subsequent diffusion of these fragments out of the cells. We used a propidium iodide-based staining procedure recently described by Telford et al. (13). Flow cytometry was carried out in an EPICS-XL (Coulter Electronics, Krefeld, FRG).

### *Flow Cytometrical Staining of the Fas-Receptor*

For immunofluorescence staining 1 x 10<sup>6</sup> cells were washed twice in phosphate-buffered saline (PBS) to remove medium. Thereafter, cells were incubated with 30 µl prediluted and FITC-labelled anti-Fas mAb UB-2 (Kamiya Biomedical Company) for 30 min at 4° C. After two wash steps in PBS immunofluorescence was quantitated by flow cytometry.

### *Glutathione Assay*

The measurement of cellular glutathione content by means of flow cytometry was performed according to Hedley (14). For appropriate comparison of glutathione content between different cell samples, untreated intact cells were gated in the forward- sideward light angle scattergram. Identical FW/SW scattergrams were used for glutathione measurement of treated cells. Therefore, the measurement of comparable cell volumes and the exclusion of cell debris which appeared outside the scattergram gate can be assured.

### *Growth Inhibition Assay*

The in vitro response to mAb CH-11 and cytostatic drugs was performed by a previously published protocol (15).

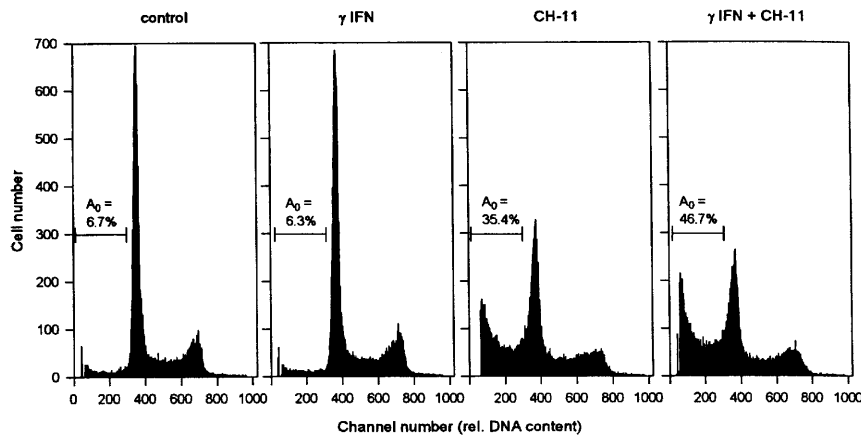
### *MTT-Assay*

MTT-assays to determine inhibition concentration 50 (IC<sub>50</sub>) values for daunorubicin, vincristine, and carboplatin have been performed as described by Kaspers et al. (16).

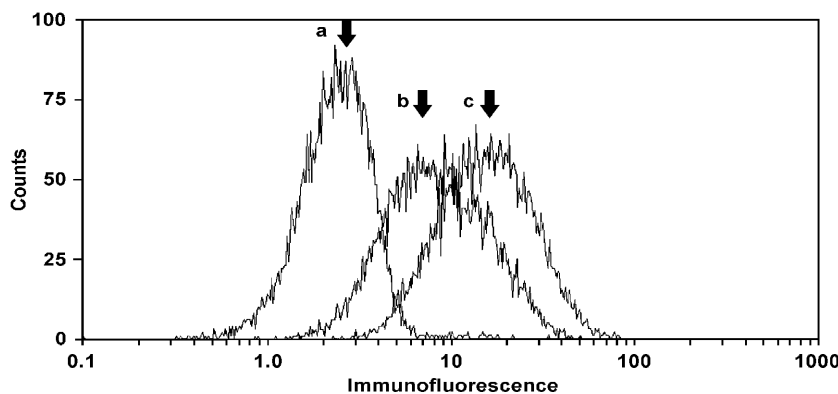
**RESULTS**

Induction of apoptosis by monoclonal antibody (mAb) CH-11 and gamma-interferon (IFN-gamma) alone or in combination was investigated in human leukemic KG-1a cells by flow cytometry. As shown in Figure 1, mAb CH-11 (1 µg/ml) triggered KG-1a cells to undergo apoptosis after 48 hrs incubation. This effect was enhanced by pretreatment of cells with IFN-gamma (100 U/ml) for 24 hrs. In contrast, very few apoptotic cells were detected in control cells or cells treated with IFN-gamma alone.

We examined whether the synergistic effect of mAb CH-11 plus IFN-gamma was due to increased Fas-receptor expression. FITC-labelled anti-Fas mAb UB-2 was used to determine Fas-receptor expression in untreated KG-1a control cells and IFN-gamma (100 U/ml) pretreated cells. Flow cytometrical measurements (Figure 2) revealed that Fas-receptor expression was nearly twice increased in IFN-gamma pretreated cells (Fig. 2, peak c) as compared to untreated KG-1a cells (Fig. 2, peak b). To calibrate Fas-receptor expression, autofluorescence of KG-1a cells was determined as control (Fig. 2, peak a).



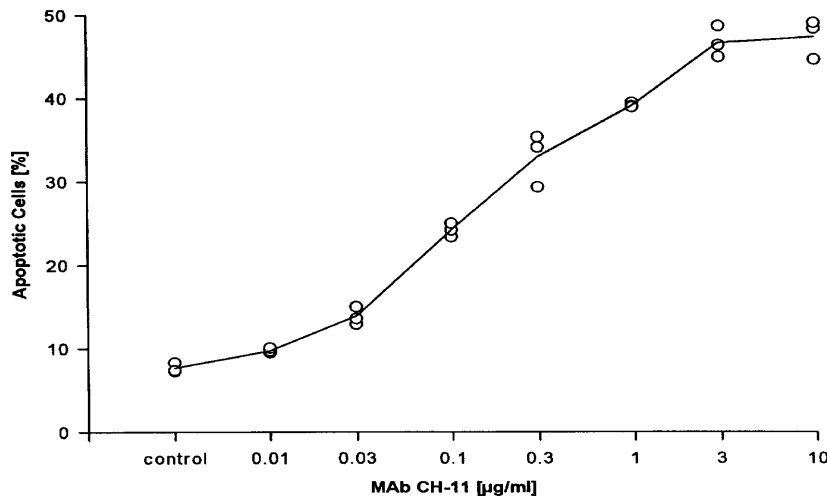
**Figure 1.** Apoptosis induced by anti-Fas monoclonal antibody CH-11 (1 µg/ml) in KG-1a cells as determined by means of flow cytometry. Gamma-IFN = gamma-interferon (100 U/ml),  $A_0$ = percent of apoptotic cells.



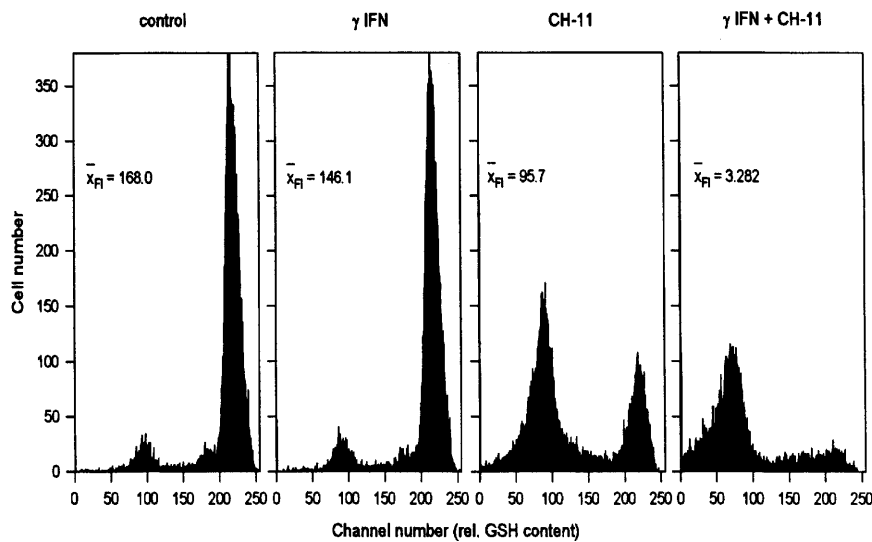
**Figure 2.** Expression of Fas-antigen in KG-1a leukemic cells as determined by mAb UB-2 and flow cytometry. Cells pretreated with 100 U/ml IFN-gamma (peak c) revealed nearly twice as much Fas-antigen expression (fluorescence mean value 15.6) than untreated cells (peak b; fluorescence mean value 8.33). Autofluorescence of unstained cells served as control (peak a; fluorescence mean value 2.41). Mean values of each 10.000 cells.

To study the dose-response relationship of the CH-11 mediated apoptosis, KG-1a cells were preincubated with IFN-gamma (100 U/ml) for 24 hrs. Subsequently, mAb CH-11 was applied in a dose range from 0.01 to 10  $\mu\text{g/ml}$  and apoptosis was measured after further 48 hrs incubation. As shown in Figure 3, induction of apoptosis in KG-1a cells by mAb CH-11 occurred in a dose-dependent manner.

The effect of mAb CH-11 +/- IFN-gamma on the cellular glutathione (GSH) level was measured by flow cytometry. Treatment of KG-1a cells with mAb CH-11 alone or together with IFN-gamma for 48 hrs resulted in an impressive depletion of GSH, whereas the GSH level of cells treated with IFN-gamma alone was only weakly affected (Figure 4).



**Figure 3.** Dose-response curve of anti-Fas mAb CH-11 mediated apoptosis in KG-1a leukemic cells. Cells were preincubated with IFN-gamma (100 U/ml) for 24 hrs. Thereafter, mAb CH-11 was applied in different concentrations and apoptosis as measured by flow cytometry after further 48 hrs incubation.



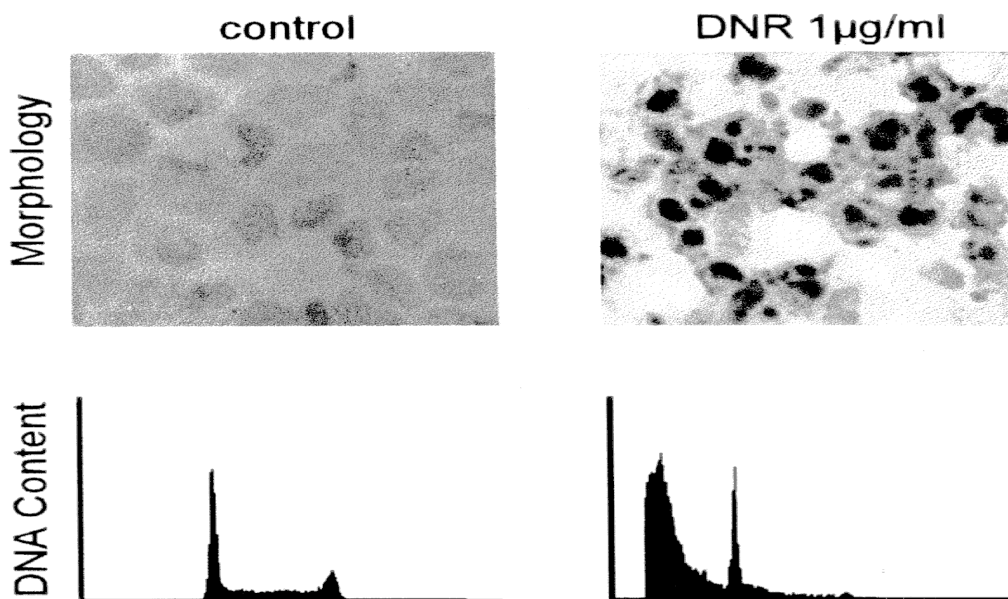
**Figure 4.** Depletion of glutathione by anti-Fas monoclonal antibody CH-11 (1  $\mu\text{g/ml}$ ) in KG-1a cells as determined by means of flow cytometry. Gamma-IFN = gamma-interferon (100 U/ml),  $X_{FI}$  = mean fluorescence.

The sensitivity of four different leukemic cell lines (KG-1a, HL-60, K562/WT, and K562/ADM) to daunorubicin (DNR), vincristine (VCR), or carboplatin (CPT) was determined by means of the MTT assay. The  $IC_{50}$  values revealed increasing resistance to DNR or VCR in the order HL-60 < K562/WT < KG-1a < K562/ADM and to CPT in the order HL-60 < KG-1a < K562/ADM < K562/WT (Table 1).

MTT assays were confirmed by morphological analysis. As shown in Figure 5, a concentration of 1  $\mu\text{g/ml}$  DNR which led to a complete inhibition of HL-60 cells in the MTT assay caused the formation of apoptosis-related fragmented nuclei. The appearance of fragmented nuclei in haematoxylin stained cell smears correlated with the appearance of apoptotic  $A_0$  cells in flow cytometry. In contrast, untreated HL-60 cells revealed intact nuclei and very few cells with subdiploid DNA content.

Table 1. Cytotoxicity of daunorubicin (DNR), vincristine (VCR), and carboplatin (CPT) on human leukemic cell lines as determined by  $IC_{50}$  values in the MTT assay. Mean values of each four determinations.

Cell Line	DNR	$IC_{50}$ Value ( $\mu\text{g/ml}$ ) VCR	CPT
HL-60	0.0333	<0.0078	5.172
K562/WT	0.1291	<0.0078	76.85
KG-1a	0.3386	0.0549	12.70
K562/ADM	10.096	0.5191	53.41

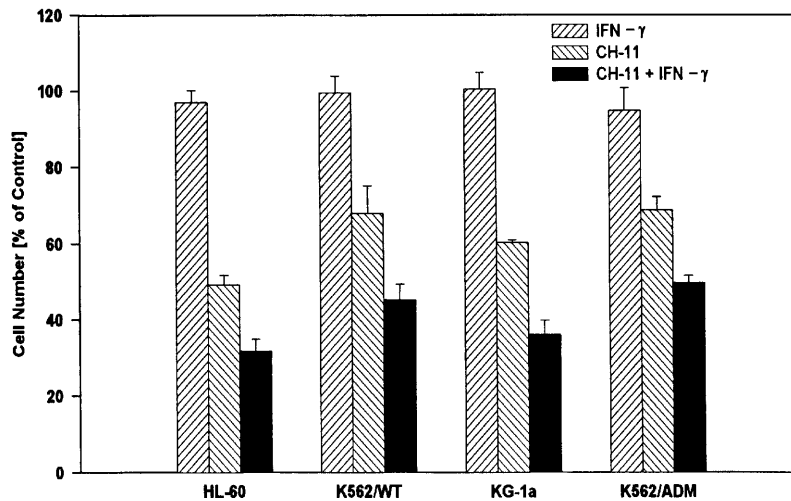


**Figure 5.** Detection of apoptotic nuclei in haematoxylin stained cell smears (upper row) and flow cytometry (lower row) after treatment of HL-60 cell with daunorubicin (1  $\mu\text{g/ml}$ ). Untreated cells served as control.

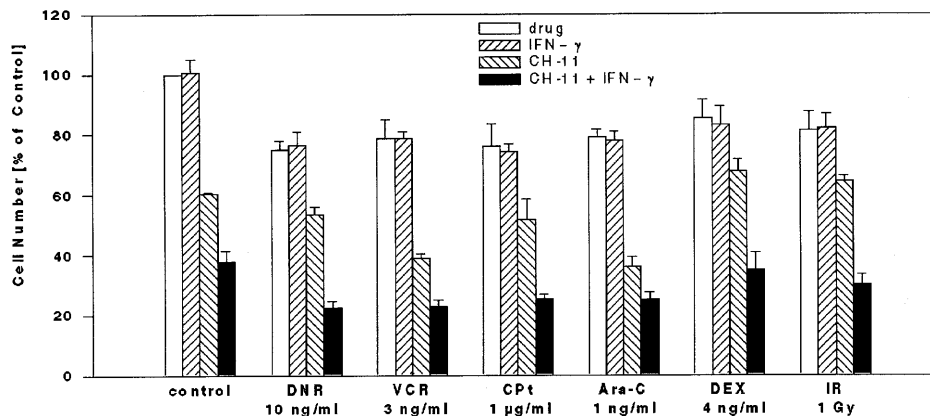
The combined application of mAb CH-11 and IFN-gamma led to inhibition of cell growth in all four cell lines with a range from 50 % to 68 % (Figure 6). These results indicate that the response to CH-11/IFN-gamma is independent from the response to DNR, VCR, or CPT in these cell lines. Since IFN-gamma alone had no effect, the combined treatment (mAb CH-11 plus IFN-gamma) is considered to be synergistic.

We further examined, whether CH-11/IFN-gamma treatment is capable to modulate sensitivity of KG-1a cells to cytostatic drugs, ionizing radiation, or dexamethasone. Dose response curves for DNR, VCR, CPT,

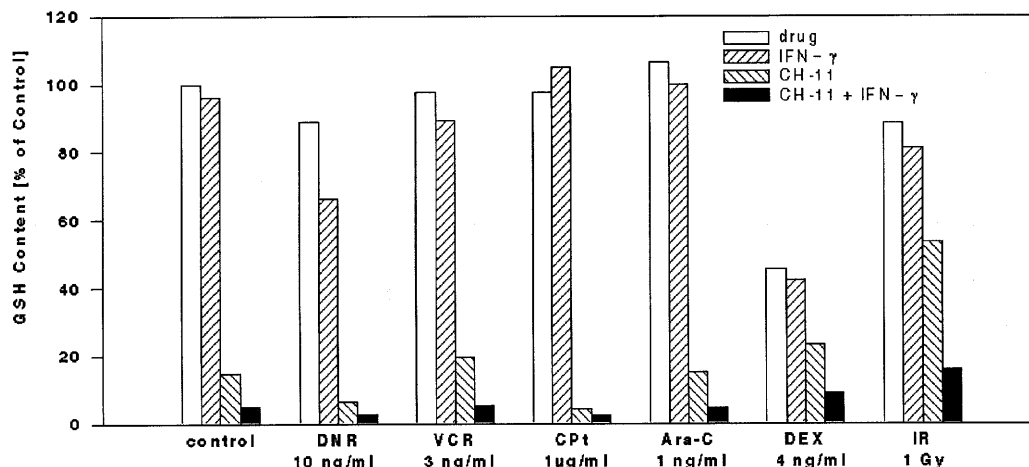
cytosine-arabioside, dexamethasone, or ionizing irradiation had been generated previously. Figure 7 shows that growth inhibition for conventional cytotoxic agents was in a range of 20-30 %. The addition of IFN-gamma had no further growth inhibitory effect, whereas the application of CH-11 +/- IFN-gamma led to increased growth inhibition (Figure 7). These effects, however, were additive rather than synergistic. Measurement of GSH levels corroborated these results. The weak depletion of GSH by conventional treatment was strongly enhanced by CH-11 +/- IFN-gamma (Figure 8).



**Figure 6.** Effect of anti-Fas monoclonal antibody CH-11 on cell growth of KG-1a, HL-60, K562/WT, and K562/ADM leukemic cell lines. Antibody CH-11 (1  $\mu$ g/ml) and gamma-interferon (IFN-gamma, 100 U/ml) were applied alone or in combination.



**Figure 7.** Effect of anti-Fas monoclonal antibody CH-11 with and without addition of cytostatic drugs, ionizing irradiation, or dexamethasone on growth of KG-1a leukemic cells. Antibody CH-11 (1  $\mu$ g/ml), gamma-interferon (IFN-gamma, 100 U/ml), daunorubicin (DNR), vincristine (VCR), carboplatin (CPT), cytosine-arabioside (AraC), dexamethasone (DEX) or ionizing irradiation (IR) were applied alone or in combination.



**Figure 8.** Effect of anti-Fas monoclonal antibody CH-11 with and without addition of cytostatic drugs, ionizing irradiation, or dexamethasone on glutathione content of KG-1a leukemic cells as determined by means of flow cytometry. Mean values of each 10,000 cells. Abbreviations see Figure 7.

## DISCUSSION

In the present investigation we found that the induction of apoptosis by anti-Fas monoclonal antibody (mAb) CH-11 is associated with a depletion of the cellular glutathione (GSH) content. GSH depletion favours the formation of free radicals and subsequent DNA strand breaks, since the GSH redox cycle is a well known detoxification pathway for the elimination of radical molecules (10). The hypothesis that anti-Fas mAb CH-11 confers cell death by radical-induced DNA strand breaks corresponds to similar results found with TNF (8). Therefore, different members of the TNF receptor gene family (TNF-R, NGF-R, Fas) may possess similar downstream signal transduction pathways leading to similar cytotoxic actions.

Since GSH has been considered as an important determinant of response to chemotherapy (10), we investigated the utility of mAb CH-11 for the treatment of multidrug-resistant (MDR) leukemic cell lines. We used two sensitive P-glycoprotein (P-gp) negative cell lines (HL-60, K562/WT) and two P-gp expressing MDR lines (KG-1a, K562/ADM). The KG-1a cell line reveals inherent resistance and expresses P-gp without prior drug selection (17), whereas the K562/ADM cell line has been selected for doxorubicin-resistance (18). We found that all four cell lines were affected by mAb CH-11 to undergo apoptosis independently of P-gp

expression and drug resistance. Thus, response of tumor cells to mAb CH-11 is independent of resistance to drugs of the MDR-type.

In conjunction with mAb CH-11, we treated KG-1a cells with cytostatic drugs, ionizing irradiation, or dexamethasone. The additional application of mAb CH-11 led to increased cytotoxicity and apoptosis. Thus, mAb CH-11 may be a suitable alternative for the treatment of tumors unresponsive to conventional treatment modalities. A clear synergism was demonstrated between mAb CH-11 and IFN-gamma, but the combination of mAb CH-11 and conventional treatment modalities were judged to be additive only. Our data are in accordance to recent results of Morimoto et al. (19) who described the combinational treatment of tumor cells with anti-Fas antibody and TNF or other drugs. There was a pleasing consistency of data obtained from MTT-assays with the determination of intracellular GSH. The molecular pathways by which mAb CH-11 mediates its cytotoxicity are poorly understood. Interestingly, Mizutani and Yoshida (9) showed that the combinational treatment of TNF-alpha with buthionine sulfoximine (BSO) enhanced cytotoxicity as compared to TNF-alpha treatment alone. BSO is a specific gamma-glutamyl-cysteine synthetase inhibitor and leads thereby to the depletion of GSH. Since the TNF-receptor and the Fas/APO-1 antigen share high amino acid sequence homology (4), both receptors may also have similar signal

transduction pathways. The involvement of GSH in TNF- $\alpha$  and mAb CH-11 mediated cytotoxicity strongly favours the hypothesis that GSH is a key molecule in the downstream effector cascade of different members of the TNF-receptor family.

Our data and the data of Morimoto et al. (19) substantiate the view that immunotherapeutical approaches might be useful adjuncts to achieve a reduction of cells in tumors resistant to conventional treatment.

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