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Anti-Fas IgG1 antibodies recognizing the same epitope of Fas/APO-1 mediate different biological effects *in vitro*

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Abstract

Fas/APO-1 is a cell surface glycoprotein that mediates programmed cell death or apoptosis when cross-linked with agonistic anti-Fas or anti-APO-1 mAb or the endogenous Fas/APO-1 ligand. In this report, we examined the *in vitro* biological properties of a panel of anti-human Fas mAb of IgG1 subclass (ZB4, VB3, WB3 and CBE). We found that anti-Fas clone VB3 induced marked apoptotic cell death in Fas/APO-1-expressing Jurkat cells, although this cell killing was delayed when compared to the cytolytic effect mediated by the prototypic anti-Fas antibody of IgM subclass (clone CH-11). The ZB4 antibody, on the other hand, efficiently blocked apoptosis induced by CH-11. The WB3 and CBE clones neither induced or inhibited apoptosis. These antibodies were all found to recognize one and the same linear site on the Fas/APO-1 molecule, despite their different biological effects. The ability of these anti-Fas mAb to induce or inhibit apoptosis appeared to correlate with their relative affinity for the Fas/APO-1 molecule. These results provide further evidence for the potential of anti-Fas antibodies of the IgG1 subclass to elicit signals via the Fas/APO-1 molecule.

Introduction

Fas/APO-1 is a transmembrane protein belonging to the nerve growth factor/tumor necrosis factor (TNF) family of receptors (1,2). Fas/APO-1 was initially characterized by mAb which induced programmed cell death or apoptosis in a variety of susceptible tumor cells (3,4). Fas/APO-1-mediated apoptosis has been implicated in several immunologically important mechanisms such as the down-regulation of immune responses (5,6) and T cell-mediated cytotoxicity (7,8).

Apoptotic signaling through Fas/APO-1 has been shown to occur upon receptor clustering on the cell surface (9). The prototypic anti-Fas mAb (clone CH-11) and anti-APO-1 mAb are of the IgM and IgG3 subclass respectively, and these mAb have been presumed to mediate their apoptotic effect through cross-linking of several Fas/APO-1 receptors, in a manner analogous to the Fas/APO-1 ligand (10,11). The anti-Fas mAb is believed to accomplish this by virtue of its multivalency and the anti-APO-1 mAb due to its inherent propensity to self-aggregate through Fc–Fc interactions (12).

For this study, anti-Fas mAb of the IgG1 subclass were generated and tested for their biological activity *in vitro*. Furthermore, the epitopes recognized by these antibodies were analyzed. The binding capacity of the mAb for the Fas/APO-1 protein was also determined. Our results show that one of the four anti-Fas mAb of IgG1 subclass utilized in this study was highly capable of inducing apoptosis, whereas an additional antibody inhibited the apoptotic process. Moreover, these different effects appear not to be mediated in an epitope-restricted manner.

Methods

Production of anti-Fas mAb

B cell hybridomas secreting anti-Fas mAb VB3, WB3, ZB4 and CBE were produced by fusing mouse myeloma NS-1 with splenocytes from mice immunized with the plasma membrane fraction of human Fas cDNA-transfected mouse T lymphoma WR19L12a cells (1). Hybridoma cell lines secreting mAb which were positive for binding to WR19L12a but not to parental mouse WR19L cells were subsequently cloned by limiting dilution. All mAb were determined to be of the IgG1 isotype.

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The original anti-Fas mAb of IgM subclass (clone CH-11) was purchased from Medical & Biological Laboratories (Nagoya, Japan).

Cells and culture conditions

The human T cell line Jurkat (kindly provided by Dr A. Slater, Karolinska Institutet) was maintained in RPMI medium supplemented with 10% FCS, 5 U/ml penicillin and 5 μg/ml streptomycin. For induction of apoptosis, anti-Fas mAb or control mAb (500 ng/ml) were added to Jurkat cells (1×10^7 cells in 1 ml culture medium) and incubation was continued for 6 h prior to cell harvesting for DNA preparation. Assessment of cell viability was performed by vital dye exclusion every 3 h for 24 h, with Jurkat cells initially seeded at 2×10^6 cells/ml. For blocking experiments, Jurkat cells were preincubated with anti-Fas IgG1 mAb or control mAb (500 ng/ml) for 2 h, and CH-11 anti-Fas mAb (500 ng/ml) was subsequently added and incubation continued for 2 h prior to harvesting of cells.

DNA fragmentation assay

Agarose gel electrophoresis of fragmented DNA was performed as previously described (13). Briefly, cells were lysed and incubated with proteinase K (1 mg/ml) for 1 h at 50°C. Following overnight ethanol precipitation and RNase treatment, the samples were extracted with phenol and chloroform:isoamylalcohol (24:1), and ethanol-precipitated again overnight. Samples were loaded onto a 1.8% agarose gel prepared in TAE buffer (40 mM Tris–acetate, 1 mM EDTA). After staining in ethidium bromide for visualization of DNA, the gels were photographed under UV illumination.

Peptide synthesis

Fifteen peptides, corresponding to the extracellular region of Fas/APO-1 (residues 16–175), were synthesized as previously described (13). The peptide amino acid sequences were derived from the amino acid sequence of the human Fas/APO-1 antigen (1). The peptides were 20 amino acids in length, each overlapping the neighboring peptide by 10 amino acids. We also synthesized additional sets of peptides of constant length in which the amino acid residues present in Fas/APO-1 peptides 11 and 12 were sequentially substituted with a glycine.

Peptide ELISA

Peptide ELISA was performed as described by Björling *et al.* (14). Briefly, microtiter plates (Titertek; Flow, MacLean, VA) were coated with 1 μg/well (for substitution sets 2 μg/well) of peptide dissolved in 0.01 mol/l carbonate buffer and then blocked with 5% BSA. The mAb were added at a concentration of 0.5 μg/well. An isotype-matched mAb directed against *Aspergillus niger* glucose oxidase (Dako, Glostrup, Denmark) was included as negative control. Peroxidase-conjugated rabbit anti-mouse Ig (Dakopatts, Glostrup, Denmark) were diluted 1:1000. Absorbance values were determined at 490 nm. For the competitive ELISA, anti-Fas mAb (1 μg/ml) were preincubated for 2 h at 37°C with 100 μg/ml of Fas/APO-1 peptides 5, 12 and 8 (the latter serving as a negative control) or in the absence of peptide, and then added to microtiter plates coated with peptide 5 as described above. Subsequent steps were carried out as before.

Flow cytometric analysis of anti-Fas binding

Jurkat cells (2×10^6) were fixed in 1% paraformaldehyde plus 0.5% fetal bovine serum for 15 min at 4°C. After washing once in PBS, cells were incubated with anti-Fas mAb (described above) or isotype-matched control mAb (Dako) at two different concentrations (0.5 and 5.0 μg/ml) for 1 h at 4°C. Cells were then washed twice with PBS and incubated for 30 min at 37°C with FITC-conjugated $F(ab')_2$ goat anti-mouse Ig (Dako) diluted 1:20 in PBS. Following incubation, cells were washed twice and resuspended in 1 ml PBS. Indirect immunofluorescence was measured using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 488 nm argon laser. Doublets and cell debris were gated out by forward scatter and side scatter, and data collected on 1×10^4 viable cells were analyzed using CellQuest software (Becton Dickinson). Fluorescence data are presented as logarithmic histograms. Kolmogorov–Smirnov statistics were employed for the analysis of the selected histograms (15).

Computer modeling of Fas/APO-1 peptides

The molecular model for the Fas/APO-1 protein was produced from the co-ordinates of the soluble human 55 kDa TNF receptor–TNF-β complex [1TNR (16)] using the Composer homology modeling program (17, 18) as implemented within the Sybyl 6.0 modeling framework (Tripos, St Louis, MO). Full details of the model and the modeling strategy will be published elsewhere (B. Fadeel *et al.*, in preparation). The peptide models were produced by excising the relevant segments from the entire Fas/APO-1 model. The peptides were then analyzed by molecular graphics using the Setor program (19) to identify regions of similarity. All calculations were performed using Sybyl 6.0 software on a Silicon Graphics Indigo^2 platform (Silicon Graphics, Mountain View, CA).

Results

Anti-Fas mAb VB3 of IgG1 subclass induces marked cell killing by apoptosis

Purified murine anti-Fas IgG1 mAb were assayed for their capacity to induce cell killing. For this purpose, we used the Fas/APO-1-expressing human leukemic T cell line Jurkat, which was previously shown to be susceptible to Fas/APO-1-mediated apoptosis (20,21). Cells were incubated for 6 h prior to harvesting and preparation of DNA, at which time cell viability, as assessed by Trypan blue exclusion, was not affected. The only exception was the CH-11-treated cultures, where apoptosis and subsequent cell death was induced very rapidly. We found that VB3 induced apoptotic cell death when added to Jurkat cells in free solution, as evidenced by DNA laddering (Fig. 1).

In order to quantify the extent of cell death, cells were cultured for prolonged periods of time and monitored for cell viability by vital dye exclusion. As shown in Fig. 2, the VB3 clone was highly efficient in killing Jurkat cells, with 40% of the cells dead within 24 h of incubation. None of the

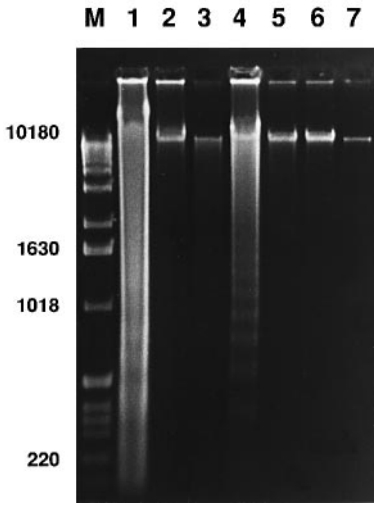

Fig. 1. Detection of apoptosis by DNA fragmentation assay in Jurkat cells treated with anti-Fas mAb. Agarose gel electrophoresis of DNA extracted from 5×10^6 Jurkat cells cultured for 6 h with 500 ng/ml of CH-11 mAb (lane 1), WB3 mAb (lane 2), ZB4 mAb (lane 3), VB3 mAb (lane 4), CBE mAb (lane 5), isotype-matched control antibody (lane 6) or with medium alone (lane 7). Oligonucleosomal DNA fragments can readily be observed in lanes 1 and 4. DNA size marker (kb) is shown in lane M.

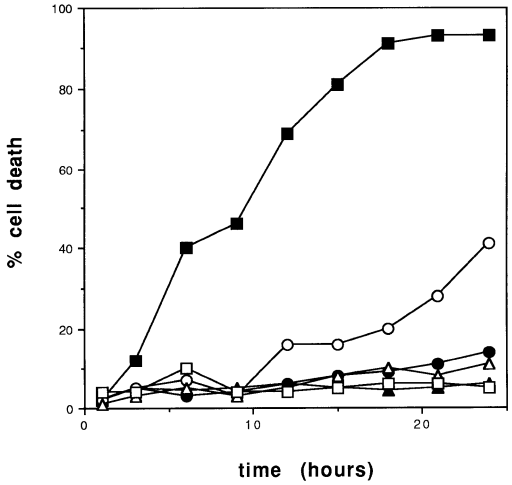

Fig. 2. Kinetics of the cytocidal effect of anti-Fas mAb on Jurkat cells. Jurkat cells (2×10^6 cells/ml) were cultured in the presence of 500 ng/ml of CH-11 mAb (filled squares), WB3 mAb (filled triangles), ZB4 mAb (filled circles), VB3 mAb (empty circles), CBE mAb (empty triangles) or with medium alone (empty squares) and cell viability was assessed by Trypan blue exclusion every 3 h for 24 h. Results shown are representative of three separate experiments.

remaining IgG1 mAb exerted any cytolytic effect. Interestingly, cell killing induced by the VB3 clone was consistently delayed by ~10 h when compared to the prototypic CH-11 clone.

We also performed a preliminary series of experiments in which secondary cross-linking antibodies were added to Jurkat cells which had been previously incubated for 2 h with

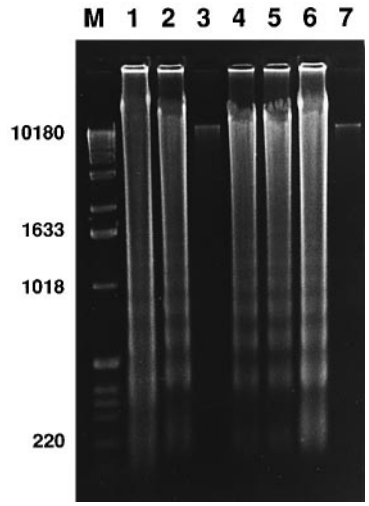

Fig. 3. Inhibition of CH-11-mediated apoptosis of Jurkat cells by the anti-Fas mAb ZB4. Jurkat cells (5×10^6) were preincubated for 2 h with 500 ng/ml of WB3 mAb (lane 2), ZB4 mAb (lane 3), VB3 mAb (lane 4), CBE mAb (lane 5) or isotype-matched control mAb (lane 6). The CH-11 mAb (500 ng/ml) was then added and incubation was continued for 2 h prior to cell harvesting and agarose gel electrophoresis of fragmented DNA. Positive control (CH-11 mAb) and negative control (medium alone) are shown in lanes 1 and 7 respectively. DNA size marker (kb) is shown in lane M.

anti-Fas mAb of the IgG1 subclass and found an enhanced cytolytic effect of anti-Fas mAb VB3 (data not shown). However, these results proved difficult to accurately reproduce using different batches of anti-Fas antibodies.

The ZB4 anti-Fas mAb functions as an antagonist of the prototypic apoptosis inducing CH-11 mAb

We also wished to assess the ability of the panel of anti-Fas mAb to inhibit apoptosis of Jurkat cells induced by the original CH-11 mAb. We found that preincubation of Jurkat cells with soluble ZB4 effectively inhibits CH-11 induced cell death at isomolar concentrations, as evidenced by the complete abrogation of DNA laddering in these cultures (Fig. 3). Neither IgG1 mAb VB3, WB3 or CBE nor the isotype-matched control antibody interfered with CH-11-induced DNA fragmentation.

Different anti-Fas mAb recognize the same linear site on the Fas/APO-1 molecule

In a previous study, we reported the epitope targeted by the CH-11 clone (13). Peptides corresponding to the antibody binding region blocked the apoptotic effect of the CH-11 mAb and to confirm the specificity of binding we tested the CH-11 mAb against selected peptides derived from this region. We have now extended the analysis and tested the IgM and IgG1 mAb for reactivity against the entire extracellular domain of Fas/APO-1, represented by linear overlapping peptides of 20 amino acids in length. We found that the different anti-Fas mAb all recognize the same peptides, albeit to a varying degree (Fig. 4A–E). Surprisingly, the mAb recognized not only the peptides corresponding to the epitope we have defined previously, but also peptide 5 (and to a lesser degree an additional peptide, designated peptide 14). The

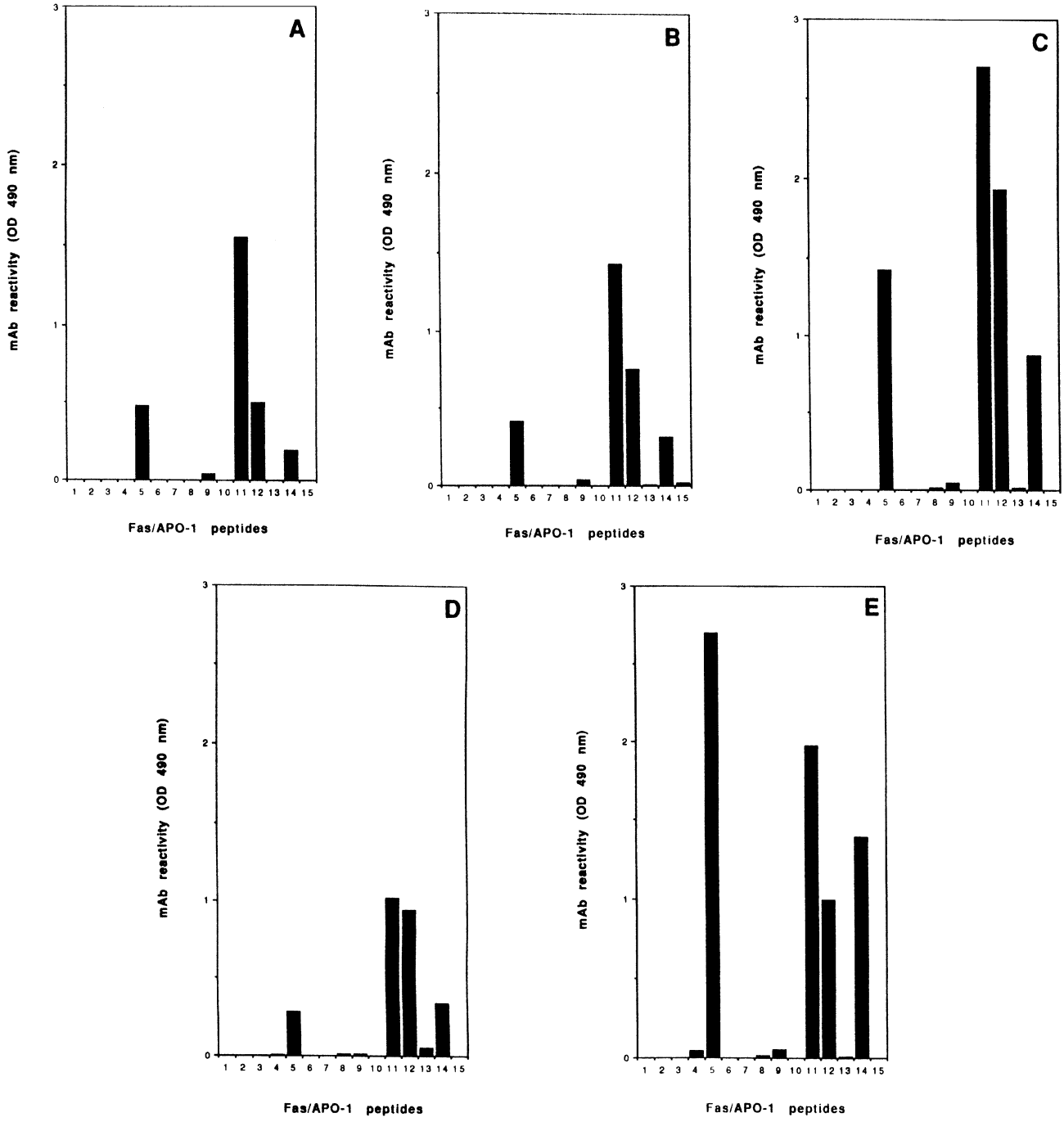

Fig. 4. Epitope mapping of anti-Fas mAb using linear peptides derived from the extracellular portion of Fas/APO-1. A panel of 15 partially overlapping peptides was tested in ELISA (see Methods) for reactivity against the CH-11 mAb (A), WB3 mAb (B), ZB4 mAb (C), VB3 mAb (D) and CBE mAb (E). The experiment was repeated twice with similar results. None of the peptides reacted to any significant degree with an isotype-matched control antibody (not shown).

two apoptosis inducing mAb VB3 and CH-11 display a similar reactivity profile, reacting to an intermediate level with Fas/APO-1 peptides 11 and 12, and only marginally with peptides 5 and 14 (Fig. 4A and D). However, the WB3 clone which exhibited no biological effect in our system also displayed such a pattern of reactivity (Fig. 4B). The levels of reactivity for the antagonistic ZB4 mAb were consistently very high, with maximum reactivity against peptides 11 and 12, and strong reactivity with peptide 5 (Fig. 4C). The CBE clone differed somewhat from the other mAb in that it reacted to a greater extent with peptide 5 compared to the other peptides (Fig. 4E).

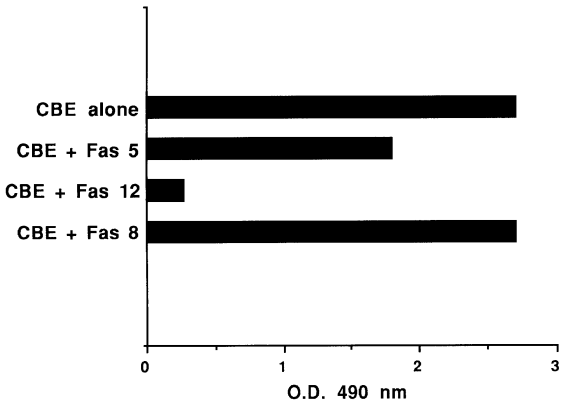

Fig. 5. Competitive ELISA with anti-Fas mAb CBE. The anti-Fas mAb CBE was preincubated for 2 h with 100 μg/ml of Fas/APO-1 peptides 5, 8 and 12 respectively or in the absence of peptide and subsequently added to a microtiter plate coated with 10 μg/ml of peptide 5. ELISA was performed as described in Methods. Peptide 12 can be seen to almost completely block binding of CBE to the immobilized peptide 5, whereas preincubation with the homologous peptide only marginally affects this binding. The negative control peptide does not influence the binding of CBE to the microtiter plate.

Anti-Fas mAb	116 E	I	N	C	T	R	T	Q	N	T	K	C	R	C	K	P	N	F	F	C 135
CH-11			N					Q	N	T			R	C		P	N	F	F	C
VB3	E		N		T		T	Q	N	T	K	C	R	C	K	P	N	F	F	C
WB3	E		N		T			Q	N	T	K	C	R	C	K	P	N	F	F	C
ZB4	E		N					Q	N	T		C	R	C	K	P	N	F	F	C
CBE	E		N				T	Q	N	T			R	C	K	P	N	F	F	C

Fig. 6. Epitope specificities of anti-Fas mAb determined with a glycine substitution set of peptides representing Fas/APO-1 peptide Glu116 to Cys135 (peptide 11). The reactivity in ELISA against the intact peptide was chosen to represent 100%. A mAb was concluded to be dependent on a particular amino acid (single letter code) when a reduction of 60% or more of the absorbance value of the substituted peptide was detected.

In order to address the issue of whether anti-Fas clone CBE recognizes a significantly different binding site from the other mAb or whether these results simply are reflective of antibody cross-reactivity, we performed a competitive ELISA assay. In this experiment, the anti-Fas clone CBE was preincubated with either Fas/APO-1 peptide 5 or 12 and subsequently added to a microtiter plate coated with peptide 5. As shown in Fig. 5, preincubation with peptide 5 only marginally reduced antibody binding, whereas peptide 12 completely blocked the binding of CBE to the microtiter plate. We conclude from these experiments that CBE, in conformity with the other anti-Fas mAb we have tested, primarily recognizes the epitope corresponding to peptides 11 and 12, with the reactivity against peptide 5 resulting from antibody cross-reactivity.

Substitution sets of peptides derived from Fas/APO-1 peptides 11 and 12 were employed to further map the binding specificities of the different mAb. The absorbance value of the mAb against the intact peptide was chosen to represent 100% and the reactivity against the substituted peptides was expressed in relation to this number. A reduction of 60% or more indicated that the deleted amino acid was important for binding of the anti-Fas mAb. These experiments showed that all anti-Fas mAb tested displayed a similar pattern of amino acid dependence, i.e. they appear to recognize the same epitope (Fig. 6).

Cross-reactivity of anti-Fas mAb may be partially explained by structural similarities of antigenic sites

The apparent cross-reactivity between peptides 5 and 14 and the partially overlapping peptides 11 and 12 observed for the anti-Fas mAb is not readily explained by amino acid similarities. Virtually no sequence homology was found upon comparison of the amino acid sequences of peptides 5, 14 and 11/12. Although these regions all contain several cysteine residues (3, 4 and 4/5 respectively), cross-reactivity is not attributable to cysteine content alone, since neither peptides 3 and 9 containing three cysteine residues each, or peptide 13 with four cysteine residues, reacted with the anti-Fas mAb (Fig. 4A–E). We recently generated a molecular model of the extracellular domain of Fas/APO-1, and have demonstrated that the regions encompassing peptides 5, 11/12 and 14 are spatially distinct and do not combine to form a conformational epitope (B. Fadeel *et al.*, in preparation). Analysis of the segments of the Fas/APO-1 model which comprise peptides 5, 11, 12 and 14 suggests that all four peptides form similar hairpin turn structures with a basic face (Fig. 7). This basic face may be recognized in the context of the peptides in ELISA. However, only peptide 11 is capable of blocking the apoptotic effect of CH-11 (13), presumably because some other required element is found uniquely in this peptide. One such feature is the pair of phenylalanine residues found only in peptide 11 (Fig. 7). Preliminary molecular dynamics simulations of the free peptides in solvent suggests that both peptides 5 and 11 may adopt stable conformations which closely resemble their structure in the native protein (C. J. Thorpe, unpublished data).

The binding capacity of anti-Fas mAb for Fas/APO-1 correlates with their in vitro effect

To examine whether the different biological effects mediated by our panel of anti-Fas mAb could be explained by different affinities for the Fas/APO-1 protein, the binding capacity of these mAb for the Fas/APO-1 protein was investigated. The anti-Fas mAb CH-11, VB3, WB3, ZB4 and CBE were incubated at high and low concentrations (5 and 0.5 μg/ml respectively) with Jurkat cells, and these cells were then stained with FITC-conjugated $F(ab')_2$ fragments of goat antibodies recognizing mouse Ig of both the IgG and IgM subclass. Indirect fluorescence was measured by flow cytometric analysis and histograms were obtained which represent the relative affinity of the different mAb for the Fas/APO-1 protein expressed on the cell surface (Fig. 8A–B). We found that the ZB4 mAb binds with a relative affinity higher than that observed for CBE and VB3. On the other hand, the binding capacity of the WB3 mAb is considerably lower when compared to the other anti-Fas mAb and this is particularly evident when low concentra-

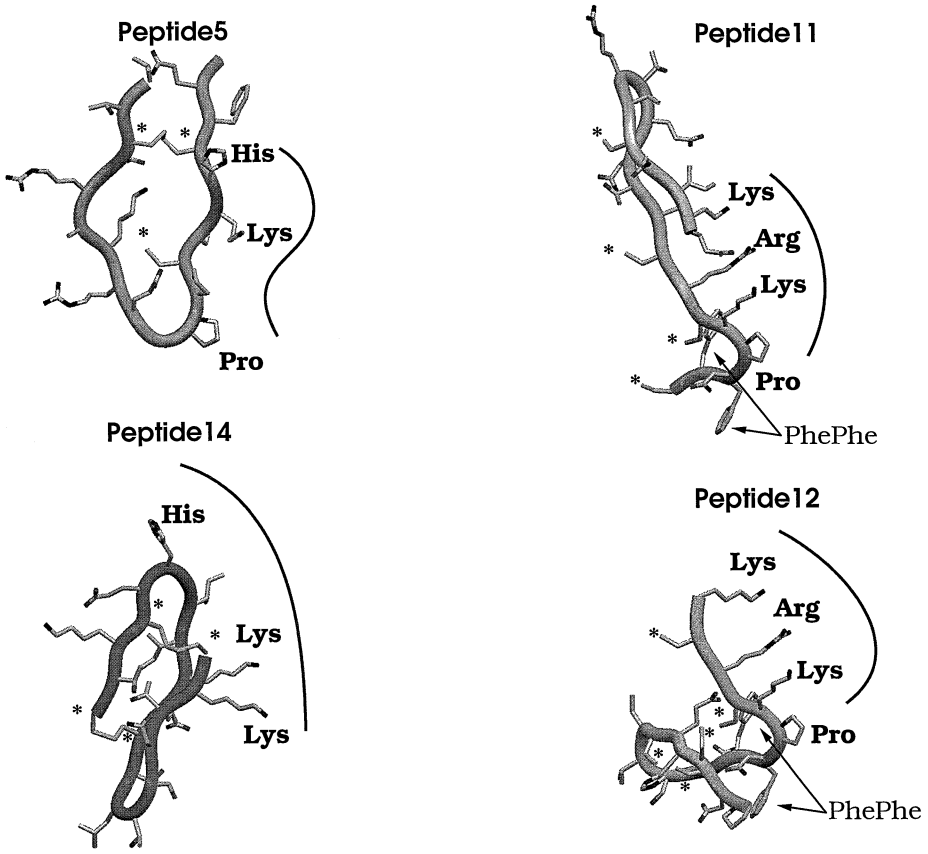

Fig. 7. Molecular graphics images of the Fas/APO-1-derived peptides 5, 11, 12 and 14. The peptides are depicted in their 'native' conformation as predicted by the Fas/APO-1 molecular model. The four peptides have an intrinsic similarity at the level of the spatial orientation of charged moieties, which may partially account for the cross-reactivity of the anti-Fas mAb. Peptides 5, 11 and 12 have a basic surface terminating with a proline residue, with peptide 14 having a similar basic surface but lacking the proline residue. The two phenylalanine residues implicated in recognition of the peptides by the anti-Fas mAb in substitution studies (Fig. 5) are arrowed in the images representing peptides 11 and 12. The presence of a cysteine residue is indicated by an asterisk. Images were produced using Setor (19).

tions of mAb are used (Fig. 8B). The binding of CH-11 was found to be similar to that of CBE and VB3, although a cautious interpretation of the latter finding is warranted, since the ability of the secondary antibody to recognize IgG and IgM antibodies respectively was not further analyzed. Kolmogorov–Smirnov statistics were employed to obtain an objective statistical measure of the differences between the histograms when compared to background levels of fluorescence (Fig. 8C). The steep decline of the slope representing the WB3 mAb indicates a low affinity for this antibody, whereas the almost level curve of the ZB4 mAb implies a much stronger binding capacity, since the relative affinity of this mAb is high even at very low antibody concentrations.

Discussion

The aim of this study was to evaluate the *in vitro* properties of a panel of anti-Fas mAb of IgG1 subclass and, furthermore, to map the epitopes targeted by these mAb. We could show that one of the IgG1 mAb tested was a highly efficient inducer of apoptotic cell death in the Fas/APO-1-expressing cell line Jurkat. Marked cell death (40%) was observed after 24 h of incubation with soluble VB3, although the kinetics of cell killing were delayed when compared to the original CH-11 mAb. These results are at variance with those obtained by Alderson *et al.* (21) who could detect only very limited cell killing effects of the anti-Fas IgG1 mAb they generated. Those antibodies were more efficient when immobilized on a solid-phase support, as opposed to adding them in free solution. Dhein *et al.* also reported that IgG1 isotype switch variants of the anti-APO-1 mAb were relatively poor inducers of apoptosis unless cross-linked (12). Thus, the IgG1 anti-Fas mAb which we have generated (clone VB3) appears to be a more potent inducer of apoptosis than those previously described. However, our findings do not imply that cross-linking of Fas/APO-1 molecules on the cell surface is irrelevant. In fact, we previously demonstrated, in agreement with Dhein *et al.*, that $F(ab')_2$ fragments of the anti-Fas mAb CH-11 were inactive unless cross-linked by secondary antibodies (22), indicating that bivalency is insufficient to induce apoptosis. However, in light of the delayed kinetics of cell killing which we have observed for the VB3 mAb, it appears that a certain lag period is required for efficient antibody self-aggregation and receptor cross-linking to occur.

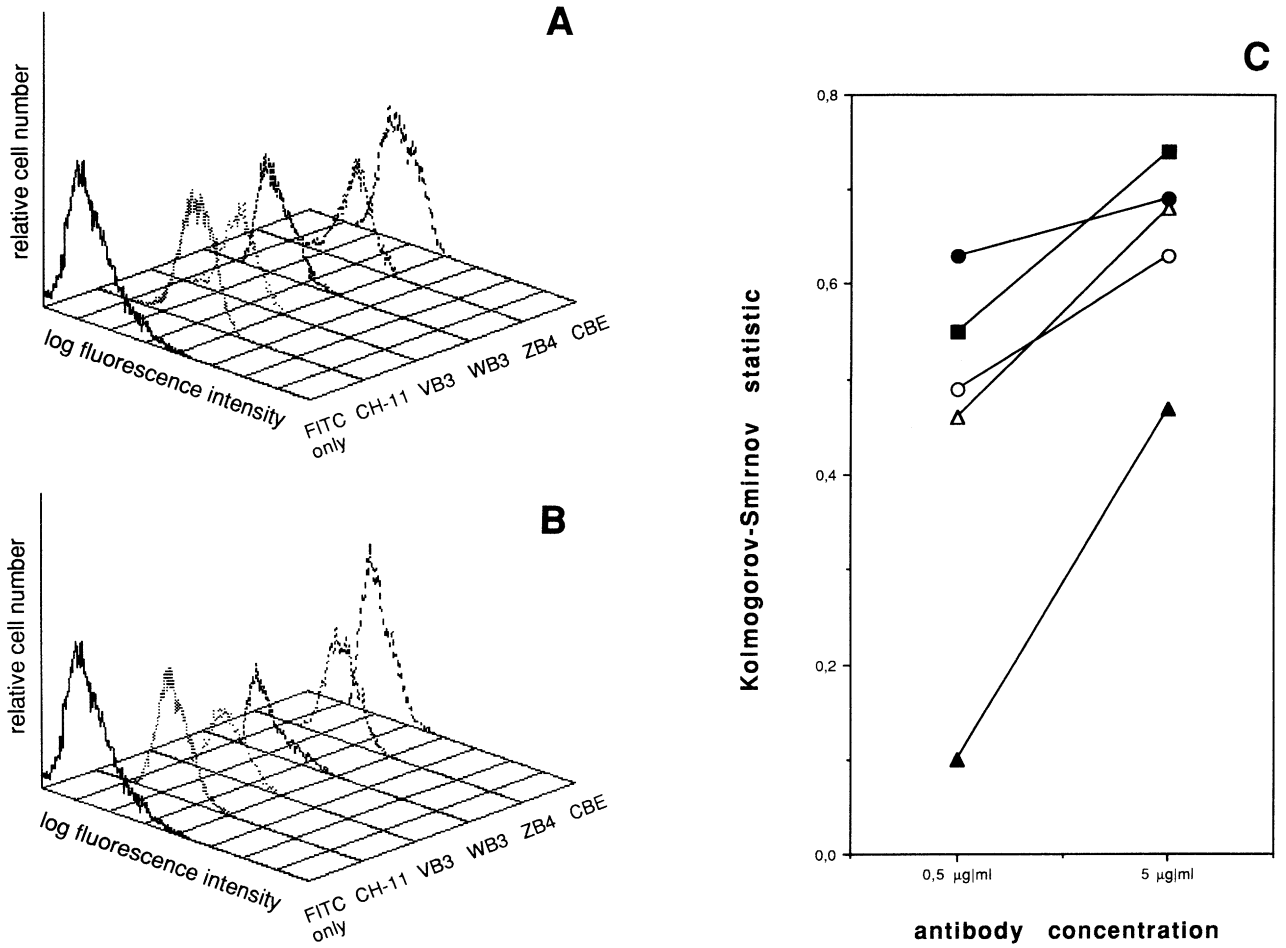

Fig. 8. Relative affinity of anti-Fas IgG1 mAb for the Fas/APO-1 protein. Jurkat cells (2×10^6) were incubated with 5.0 µg/ml (A) and 0.5 µg/ml (B) of anti-Fas mAb and subsequently labeled with FITC-conjugated $F(ab')_2$ fragments of goat anti-mouse Ig. Indirect fluorescence was measured and histograms were obtained which represent the relative affinity or binding capacity of the respective anti-Fas mAb for the membrane bound Fas/APO-1 protein. (C) The histograms obtained for anti-Fas mAb CH-11 (filled squares), WB3 (filled triangles), ZB4 (filled circles), VB3 (empty circles) and CBE (empty triangles) were compared with the histogram derived from Jurkat cells stained with only FITC-conjugated secondary antibody, and the Kolmogorov–Smirnov statistic, i.e. the greatest difference between the two curves, was calculated. Note the marked difference in the gradient of the curve representing anti-Fas mAb ZB4 and WB3.

We also evaluated the panel of anti-Fas mAb with regard to blocking apoptosis induced by the prototypic CH-11 anti-Fas mAb. The ZB4 clone, which we could show recognizes the same epitope as CH-11 (see below), had the capacity to inhibit CH-11-induced apoptosis at isomolar concentrations in a short-term culture system. It would appear, therefore, that individual antibodies of the IgG1 subclass, such as ZB4, may occupy the antibody binding site on the Fas/APO-1 molecule, and thereby abrogate the binding and subsequent receptor clustering induced by the anti-Fas IgM mAb (10,13). Alderson *et al.* also described certain anti-Fas mAb which inhibited CH-11, presumably by binding to an epitope distinct from that recognized by the CH-11 antibody (21). However, epitope mapping was not performed for the mAb used in that report. Whereas these findings may simply reflect different properties of our respective panels of anti-Fas mAb, they may also be compatible with different levels of affinity for the same epitope.

We have recently mapped the epitope targeted by the prototypic anti-Fas mAb CH-11, and found this to be a linear site situated between the second and third cysteine-rich domains on the extracellular portion of Fas/APO-1 (13). In the present study, we employed the same peptide-based strategy in an attempt to map the epitopes recognized by our panel of IgG1 mAb. We found that the IgG1 mAb all recognized the same linear site (corresponding to amino acids 126–135) as the prototypic CH-11 mAb, regardless of whether they induced (VB3) or inhibited (ZB4) apoptosis or exerted no effect (WB3 and CBE) in our system. In this context, one may note that the mutated Fas/APO-1 molecules in patients with autoimmune lymphoproliferative syndrome described by Fisher *et al.*, which lacked the epitope we have defined, could be detected neither by the ZB4 nor the CH-11 mAb (23). However, surface expression of this mutant protein could be detected with a rabbit polyclonal antibody recognizing amino acids 21–38 (corresponding to Fas/APO-1 peptide 1 in the present study). This is in accordance with our findings that both CH-11 and ZB4 recognize the same region on the Fas/APO-1 molecule.

The finding that different mAb may recognize the same

epitope and elicit different effects (or no effect at all) appears somewhat puzzling. One explanation is that such antibodies may bind the same *antigenic region*, whereas within this region there could be several (overlapping) epitopes, as described for instance for anti-HIV-1 and anti-SIV_{mac} mAb (24), i.e. the mAb may bind within the same region but in a slightly different manner thus exerting different effects. However, these arguments do not appear to hold true with regard to our anti-Fas mAb, since the substitution set ELISA experiments revealed an identical pattern of amino acid dependency for the different antibodies. Could these different outcomes of the antibody–antigen interaction be accounted for by different affinities of the mAb for the same epitope? Our results are consistent with the view that very strong binding will confer antagonistic properties to the antibody (ZB4), while an optimal affinity for the antibody binding epitope is associated, at least in some cases, with an apoptotic effect (CH-11 and VB3). On the other hand, weak antibody affinity for this epitope appears insufficient for mediating any biological effect (WB3). The recent proposal for serial triggering of TCR by the same peptide–MHC complex (25) provided an elegant explanation for the high sensitivity–low affinity paradox of T cell antigen recognition and gives an example of the critical importance of receptor–ligand affinities which are neither too strong or too weak. Fast off-rates are not unique to TCR interactions; similar low affinities and fast dissociation rate constants have been described for cell adhesion molecules that mediate transient intercellular adhesion (26). Indeed, it is tempting to speculate whether serial engagement might also be a mechanism for Fas/APO-1-mediated apoptosis; conversely, one may consider a role for receptor clustering in the TCR system. It is reasonable to assume that receptor binding precedes self-aggregation of anti-Fas antibodies (12,27) and under such conditions an antibody of the IgM isotype may be surmised to hold the upper hand; IgG mAb would require more time for binding and reaggregation to occur following detachment from the first receptor complex. Such a scenario may thus provide a tentative explanation for the 10 h delay in cytolytic effect which we observed for the IgG1 mAb.

Our panel of anti-Fas mAb reacted not only with the epitope corresponding to amino acids 126–135, but also with a second site which is located distally to the first epitope and shows no sequence homology with this region. Although antibodies are highly specific, cross-reactions with closely related antigens occur frequently. For instance, the anti-hen egg lysozyme mAb D11.15 was found to cross-react extensively with several avian lysozymes, due to binding of a common or 'public' epitope shared by the different antigens (28). However, Fas/APO-1 peptides 5 and 11/12 (and 14) display virtually no sequence homology. On the other hand, molecular modeling of these peptide segments can be used to partially explain the cross-reactivity of the anti-Fas mAb. All of the peptides have a set of basic residues along one face, a conserved proline at the tip of the loop and potentially similar disulfide bonds. Although in peptide 11 there is a different disulfide connectivity to peptide 5, this connectivity is imposed by the environment in the protein structure. In solution as short peptides, the pattern of disulphide bridges demonstrated by peptide 5 is more stable and thus may be adopted by both peptides as the spacing of the cysteine residues is similar. Cross-reactivity of these mAb may thus be dependent on structural similarities even in the absence of sequence homology. Alderson *et al.* could show that some anti-Fas mAb bound to membrane bound Fas/APO-1, without exerting any apoptotic or inhibitory effect; these antibodies were thus concluded to recognize epitopes not involved in signaling (21). Such an epitope might be the highly antigenic region corresponding to peptide 5 in our study.

In conclusion, we have demonstrated highly efficient cell killing of Fas/APO-1-expressing cells by an IgG1 anti-Fas mAb. Cell death is delayed when compared to death induced by the prototypic CH-11 mAb, possibly reflecting the additional time needed for self-aggregation and receptor clustering to occur. We also mapped the epitopes targeted by the mAb and found that the different biological effects (induction or inhibition of apoptosis) elicited by this panel of anti-Fas mAb appeared not to be epitope restricted. However, the relative affinity for the Fas/APO-1 protein, as determined by FACS analysis, correlated to some extent with the biological effect of the mAb. These observations extend our knowledge of the mechanisms of Fas/APO-1 signaling by different classes of anti-Fas antibodies.

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Abbreviations

TNF tumor necrosis factor

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