Expression of conformationally constrained adhesion peptide in an antibody CDR loop and inhibition of natural killer cell cytotoxic activity by an antibody antigenized with the RGD motif

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Communicated by P.A. Cazenave

We report that an antibody engineered to express three Arg-Gly-Asp (RGD) repeats in the third complementarity-determining region of the heavy chain (antigenized antibody) efficiently inhibits the lysis of human erythroleukemia K-562 cells by natural killer (NK) cells. Synthetic peptides containing RGD did not inhibit. Inhibition was specific for the (RGD)₃-containing loop and required simultaneous occupancy of the Fc receptor (CD16) on effector cells. The antigenized antibody inhibited other forms of cytotoxicity mediated by NK cells but not cytotoxicity mediated by major histocompatibility complex-restricted cytotoxic T lymphocytes (CTL). A three-dimensional model of the engineered antibody loop shows the structure and physicochemical characteristics probably required for the ligand activity. The results indicate that an RGD motif is involved in the productive interaction between NK and target cells. Moreover, they show that peptide expression in the hypervariable loops of an antibody molecule is an efficient procedure for stabilizing oligopeptides within a limited spectrum of tertiary structures. This is a new approach towards imparting ligand properties to antibody molecules and can be used to study the biological function and specificity of short peptide motifs, including those involved in cell adhesion.

Key words: antibody engineering/antigenized antibody/cytotoxicity/NK cells/RGD

Introduction

Human natural killer (NK) cells are a discrete subpopulation of lymphocytes that lack clonally distributed antigen receptors. Their cell-surface phenotype is CD3[−], CD16.2/FcγRIII.2⁺, ζ⁺ and CD56⁺ (Qiu *et al.*, 1990; Lanier and Phillips, 1992). NK cells lyse tumor and virus-infected cells (Trinchieri, 1989; Lewis and McGee, 1992; Welsh and Vargas-Cortes, 1992) without previous antigen

sensitization and independently of major histocompatibility complex (MHC) restriction. They are considered a phylogenetically primitive barrier system against transformed cells and cells infected by viruses or parasites (Trinchieri, 1989).

Interactions between NK cells and target cells begin with scanning and recognition of target cells. These are followed by adhesion, formation of effector–target (E:T) conjugates and lysis of the target cells. Signal transduction and activation of NK cells involve at least two molecules: CD16 and CD2. CD16 (FcγRIII.2) associates with the ζ chain (Lanier *et al.*, 1989; Anderson *et al.*, 1990) to form a complex equivalent to the T-cell receptor (TCR)–CD3 complex in T cells. CD2 is the ligand for CD58 (LFA-3) (Siliciano *et al.*, 1985). CD56 (N-CAM), whose expression correlates with activation of NK cells (Hellstrand *et al.*, 1991), appears to be involved in the recognition of allogeneic targets (Suzuki *et al.*, 1991), but its role in homophilic interactions is disputed (Lanier *et al.*, 1991). Results of blocking studies with monoclonal antibodies (Dongworth *et al.*, 1985; Mentzer *et al.*, 1986; Axberg *et al.*, 1987; Springer *et al.*, 1987; Robertson *et al.*, 1990; Timonen *et al.*, 1990) suggest that other adhesion molecules are also involved, e.g. CD54 (ICAM-1), CD18/CD11a (LFA-1), CD18/CD11b (MAC-1, CR3) or CD18/CD11c (p150/95). NK cells also express fibronectin and laminin (Schwarz and Hiserodt, 1988; Santoni *et al.*, 1989), and specific antibodies to fibronectin inhibit the cytotoxic effects of NK cells without affecting formation of E:T conjugates.

Adhesion plays an important role in the function of the immune system and apparently involves a large number of molecules, including ones that contain the Arg-Gly-Asp (RGD) tripeptide (Springer, 1990). The hydrophilic RGD sequence is a motif involved in a variety of adhesion processes (Rixon *et al.*, 1983; Ginsberg *et al.*, 1985; Hayman *et al.*, 1985; Ruoslahti and Pierschbacher, 1986), and it serves as the binding site for several integrins (Hynes, 1992). Involvement of RGD in specialized activities of NK cells remains unclear. Previous studies showed that synthetic peptides containing RGD do not inhibit adhesion of NK cells to target cells or NK cell cytotoxicity (Phillips *et al.*, 1991; Timonen *et al.*, 1990). However, this could simply reflect an intrinsic inability of synthetic peptides to meet appropriate conformational and/or structural requirements, leaving unanswered the question as to whether RGD plays any role in the function of NK cells.

A better molecular definition is a prerequisite for understanding the function of active sites in protein antigens, ligands and receptors and for designing ways to manipulate the immune system. Synthetic peptides have helped in this process (Atassi, 1980; Lerner, 1983; Geysen, 1985). However, their intrinsic lack of stable tertiary structure limits their use in studies of the molecular requirements of ligand function and ligand–receptor interactions. Methods to improve the conformational stability of synthetic peptides

include modification of the amide backbone, cyclization, mimicking β -turns and using unusual amino acids. As an alternative to these approaches, we have developed antigenization of antibody, a new procedure for stabilizing oligopeptides within a limited spectrum of tertiary structures (Zanetti, 1992). Oligopeptide epitopes are expressed in the molecular environment of the hypervariable loops of an antibody molecule in such a fashion that the peptides acquire an ordered conformation. Antigenized antibodies (A^g Ab) can be used as conformational mimics of antigens and ligands.

We previously engineered an A^g Ab in which the hydrophilic tetrapeptide Asn-Ala-Asn-Pro (NANP) of the circumsporozoite protein of *Plasmodium falciparum* was added to the third complementarity-determining region (CDR3) of an antibody heavy (H) chain (Billetta *et al.*, 1991). Within the antibody loop the malarial peptide acquired a three-dimensional conformation that approximated immunologically the conformation of the corresponding native antigen (Sollazzo *et al.*, 1990a). Immunization with the A^g Ab induced antibodies that blocked *in vitro* *P.falciparum* infection of human hepatocytes (Billetta *et al.*, 1991). We used a similar approach and constrained RGD sequences within the CDR3 of the H chain of an antibody to show that this can effectively stabilize the RGD motif so that the A^g Ab may acquire ligand properties absent in synthetic RGD peptides. Our results indicate that expression of RGD in an antibody β -loop is sufficient to impart RGD-dependent ligand properties to this molecule, e.g. inhibition of tumor cell adhesion to fibronectin (R.Billetta, P.Lanza, F.Rossi and M.Zanetti, manuscript in preparation), which allowed us to analyze and establish a role for the RGD motif in the function of NK cells.

Results and Discussion

Antigenized antibodies with RGD loops

Previous studies on the RGD tripeptide in natural proteins such as fibronectin (Main *et al.*, 1992), tenascin (Leahy *et al.*, 1992) and several disintegrins (Adler *et al.*, 1991; Saudek *et al.*, 1991) indicated that this short hydrophilic peptide sequence exists at the tip of exposed β -loops. On the basis of this information, we decided to engineer the RGD amino acid motif in the CDR3 of an antibody molecule. We used the hydrophilicity index (Kyte and Doolittle, 1982) as an indicator of surface exposure of RGD. We compared the index of the engineered loop with the indexes of two RGD-containing proteins for which structural information is available: fibronectin type III domain (Leahy *et al.*, 1992; Main *et al.*, 1992) and echistatin, a disintegrin isolated from snake venom (Gan *et al.*, 1988) (Figure 1, panels a and b). As indicated in panels c and d, the profiles are consistent with the prediction that CDR3 loops expressing RGD would be hydrophilic and exposed on the surface of the molecule, with the effect being more pronounced for three RGD repeats than for one RGD. We chose to engineer the CDR3 of the H chain for three reasons: (i) a predicted model of the CDR3 of the parent antibody indicated that this loop is exposed on the surface of the molecule (Sollazzo *et al.*, 1990b), (ii) CDR3 is the most variable in length and composition among the six antibody hypervariable regions (Kabat *et al.*, 1987), (iii) CDR3 interconnects two adjacent β strands, and (iv) we had already successfully expressed in the same antibody molecule a hydrophilic peptide (NANP) that met both

Fig. 1. Hydrophilicity profiles of RGD-containing polypeptides. (a) Hydrophilic profile of the disintegrin, echistatin, between amino acid residues 14 and 36. (b) Type III domain of the extracellular matrix protein fibronectin between amino acid residues 68 and 90. (c and d) A^g Ab γ_1 RGD and γ_1 (RGD)₃ (amino acid residues 92–114 and 92–120, respectively of the H chain). The A^g Ab constructs are flanked by two Val-Pro (VP) doublets that correspond to residues 100–101 and 105–106. RGD residues are shown in bold and underlined.

antigenicity (Sollazzo *et al.*, 1990a) and immunogenicity (Billetta *et al.*, 1991) requirements.

Figure 2 panels a and b illustrate the structure of the engineered H-chain genes containing the RGD or (RGD)₃ sequences and the general configuration of the two molecules [referred to as γ_1 RGD and γ_1 (RGD)₃]. The site of insertion of the hydrophilic sequences in CDR3 is shown. The structure and H₂L₂ composition of the A^g Ab molecule ensuing from transfecting murine J558L myeloma cells is also depicted (Figure 2c and d). After purification, the proper polypeptide composition was checked by using SDS-PAGE. Both molecules, γ_1 RGD and γ_1 (RGD)₃, had an apparent molecular weight of 165 kDa and after reduction resolved into two polypeptide bands of 55 and 25 kDa, respectively (data not shown).

Structure of the engineered loops containing RGD

Conformational models for the two engineered variable regions were constructed, refined through a combination of energy minimization and molecular dynamics and analyzed (Figure 3). Both RGD-containing loops project outwards from the main body of the protein, ensuring that they are fully accessible for binding to a putative acceptor molecule. A comparison of panels a and d shows that the

Fig. 2. Models for the structure of A8Ab expressing RGD. (a and b) Diagram of pN γ_1 RGD (a) and pN γ_1 (RGD)₃ (b) expression vectors. The coding strand of CDR3 is shown with the RGD-coding sequence in parenthesis. (c and d) The expressed A8Abs γ_1 RGD and γ_1 (RGD)₃ are mouse/human chimeras. The H chain is the product of the fusion of a human γ_1 C region with the parental V_H murine engineered to express one (c) or three (d) RGD repeats. As shown, the D region was modified by insertion of RGD or (RGD)₃ between 94V and 95P. The inserted sequences are flanked by a VP doublet [VP(RGD or RGD₃)VP]. The light (L) chain is the murine λ_1 provided by the myeloma J558L host cell (H and L chains not to scale). B, BamHI; RI, EcoRI; Neo, neomycin resistance; Amp, ampicillin resistance.

(RGD)₃-containing loop extends much further into solvent. All low energy conformations of the models of the (RGD)₃ loop appear to be quite similar, indicating that each loop has a fairly rigid structure, an effect probably due to residue Arg102, which is highly strained in the model, being crowded by the main body of the protein, mostly the H chain. The backbone structure of the loop also constrains the charged residues to be quite far apart (data not shown), so that oppositely charged side chains do not intertwine to neutralize any long-range electrostatic effect.

Further analysis of the molecular model indicated that oligomerization of RGD in the antibody loop enhanced surface accessibility but also created interesting electrostatic profiles. Side views of the RGD and (RGD)₃ loops (Figure 3b and e) show their highly polar character. RGD is mostly negative, and (RGD)₃ has an alternating positive and negative pattern with a prevalently positive N-terminal region and a prevalently negative C-terminal region. This difference is most apparent when the loops are viewed from the top (Figure 3c and f). This is probably the electrostatic profile presented to a putative surface acceptor molecule approaching from a distance. Interestingly, even in the shorter loop, the side chains of Arg102 and Asp104 point away from each other, implying the existence of a dipole. Although this was not reflected in the electrostatic field shown in Figure 3c, the dipole may be lost in the field for the rest of the protein, and the effect can be seen only when the loop is large enough to project away (Figure 3e). Thus, in the unbound state, the (RGD)₃ loop contains both positively and negatively charged residues in proximity.

Inhibition of NK cell cytotoxicity

The two A8Abs were tested in a conventional ⁵¹Cr release assay with nonadherent human peripheral blood leucocytes (PBLs) as effector cells and the human erythroleukemia cell line K-562 as target cells. Antibody γ_1 (RGD)₃ but not synthetic peptides RGDS and GdRGDSP inhibited the cytotoxic effects of NK cells (Figure 4). The inhibition by

γ_1 RGD was weaker than that caused by γ_1 (RGD)₃ and was noticeable only at an E:T ratio of 50:1. Control antibody γ_1 NANP, a recombinant antibody that differs only in the chemical composition of the hydrophilic peptide in CDR3 (three Asn-Ala-Asn-Pro repeats) (Billetta *et al.*, 1991), did not inhibit the cytotoxic effects of NK cells. On the basis of these results, further experiments were done with γ_1 (RGD)₃. Inhibition by γ_1 (RGD)₃ was concentration dependent (Figure 4, inset), with a plateau at 100 μ g/ml. We ruled out the possibility that inhibition was merely due to a reciprocal effect of NK cells on each other (autocytotoxicity) that could have limited their availability. In fact, ⁵¹Cr-labelled NK cells did not undergo lysis in the presence of γ_1 (RGD)₃ (data not shown). These results indicate that the inhibitory effect is specific for the RGD-containing loops and suggest that stabilization of the tertiary structure of the RGD motif is required for inhibition.

To confirm the involvement of RGD, we prepared conventional anti-idiotypic (Id) antibodies specific for the (RGD)₃ loop. Effector cells preincubated with anti-Id antibodies, but not immunoglobulins of the preimmune serum of the same animal, lost almost all lytic activity (Figure 5). We also investigated the possible role of CD16, the type III low-affinity IgG receptor (Fc γ RIII) expressed on NK cells. When effector cells were preincubated with a monoclonal antibody to CD16, the inhibitory activity of γ_1 (RGD)₃ was greatly diminished (Figure 6a). As shown, the NK cell activity *per se* was not affected by the anti-CD16 antibody. The role of CD16 was further addressed by using the following two approaches. First, we used three CD56⁺/CD2⁺/CD3⁻ NK clones, two of which did not express CD16 (Bacchetta *et al.*, 1993). As shown in Figure 6b, γ_1 (RGD)₃ inhibited the lytic activity of the CD16⁺ clone J104, but not that of the CD16⁻ clones J40 and J201 (Figure 6b). Second, we used Fab and F(ab')₂ fragments of γ_1 (RGD)₃. At a molar concentration higher than that sufficient for maximal inhibition by the intact antibody, neither the Fab nor the F(ab')₂ fragments caused

Fig. 3. Molecular models of the engineered V regions RGD and (RGD)₃. The RGD-containing molecule is shown in the left panels and (RGD)₃ in the right panels. The L chain is pink, and the H chain is green. The loops are yellow except for Arg (blue), Gly (white) and aspartic acid (red). Panels **a** and **d** depict the molecules as solid ribbons through the backbone atoms with the (RGD)₃ loop projecting much further from the main body of the V region than RGD. Both loops are fairly rigid, as shown by high-temperature molecular dynamics, and are roughly planar. The sidechains of the loop residues point away from each other, accentuating the dipolar character of the loops. Panels **b–d** and **e–f** show contours of the electrostatic fields of the two molecules. Panels **b** and **e** are side views of the RGD and (RGD)₃ loops, respectively. Panels **c** and **f** are top views. Positive contours are shown in blue and negative ones in red. Contour values are ± 0.01 , ± 0.02 , ± 0.03 , ± 0.05 , ± 0.07 , ± 0.1 and ± 0.15 kT/e. In the side view, RGD is largely negative, and (RGD)₃ has alternating positive and negative lobes. However, (RGD)₃ is also more positive at the N-terminal end of the loop and negative at the C-terminal end. In the top views, RGD is still largely negative, presumably because of the proximity effects of the rest of the protein. On the other hand, (RGD)₃ is markedly dipolar in this view.

any inhibition (Figure 6c). Collectively, these data indicate that inhibition by $\gamma_1(\text{RGD})_3$ involves the (RGD)₃ loops and requires the simultaneous occupancy of CD16 on effector cells. This and the lack of stable conformation may explain why synthetic peptides are not inhibitory.

Cytotoxic activity of NK cells and CTLs

One implication of our results concerns cytotoxicity in general. Cytotoxic effects can be mediated by a variety of cells and mechanisms, all leading to the destruction of target cells. NK cells, lymphokine-activated killer (LAK) cells (Perussia, 1991) and killer cells that lyse target cells via an antibody specific for an antigen expressed at the surface of the target cell (ADCC) (Reynolds and Ortaldo, 1990) all lyse irrespective of the MHC of the target cells and without using a clonally distributed receptor for antigen. On the other hand, MHC-restricted and antigen-specific cytotoxic T lymphocytes (CTLs) express clonally distributed receptors for antigen and lyse targets that present processed antigen peptides in the context of MHC gene products (Berke, 1991).

Although the most common type of CTLs expresses the CD8 molecule and recognizes antigen peptides presented in the context of class I MHC gene products, other CTLs express the CD4 molecule and recognize antigen peptides presented in the context of class II MHC gene products (Barnaba *et al.*, 1989; Orentas *et al.*, 1990).

We sought to analyze the aforementioned types of cytotoxicity by using $\gamma_1(\text{RGD})_3$. The results (Figure 7) indicate that whereas the $\text{A}^\text{g}\text{Ab}$ blocked the three kinds of cytotoxic activity that involve, directly or indirectly, NK cells as the effector cells, it had no effect on conventional MHC-restricted cytotoxic effects mediated by CTLs. As indicated (panels a–c), $\gamma_1(\text{RGD})_3$ inhibited in a dose-dependent manner NK and LAK cells as well as ADCC. Interestingly, a discrete inhibition of ADCC also occurred with the control engineered antibody; this may reflect the critical role of CD16 in the effector phase of ADCC. As for NK cells, inhibition of ADCC by $\gamma_1(\text{RGD})_3$ was reversed by a monoclonal antibody to CD16 (data not shown). In contrast, no inhibition was found either with CD8⁺ or CD4⁺ CTL clones (panels d and e).

Fig. 4. Inhibition of NK cell cytotoxic activity by A&Abs expressing RGD. Cytotoxic activity was determined in the presence of the following inhibitors: A&Abs $\gamma_1(\text{RGD})_3$, $\gamma_1\text{RGD}$ and $\gamma_1\text{NANP}$ (as a negative control) and synthetic peptides Arg-Gly-Asp-Ser (RGDS) and Gly-D-Arg-Gly-Asp-Ser-Pro (GdRGDSP). The inset shows the dose dependency of the inhibition by $\gamma_1(\text{RGD})_3$ (□). $\gamma_1\text{NANP}$ (■) served as a control. Results refer to a representative experiment and are expressed as percent lysis $\pm$ SD. NK cell lysis in the absence of inhibitor is indicated by the black bar.

Fig. 5. Involvement of an RGD motif on NK cells. Pretreatment of effector cells with rabbit anti-idiotypic (anti-Id) antibodies to the $(\text{RGD})_3$ loop (○) block lysis of K-562 cells. Protein A-purified immunoglobulins from the preimmune serum of the same animal (●) served as a control. (⊕) denotes NK cell cytotoxicity in the absence of antibody.

Implications for the cytotoxic phenomenon

NK cells and CTLs have similar mechanisms of cytotoxic activity. In both cases, binding to target cells is rapidly followed by the delivery of a lethal hit associated with the release of lytic cytokines. Our results reveal, however, a subtle difference between NK cells and CTLs, i.e. resistance of CTLs to the inhibitory activity of $\gamma_1(\text{RGD})_3$. It is tempting to speculate that NK cells, a more primitive form of cytotoxic cells, may base their recognition system on conserved structural motifs such as RGD. This is in sharp

Fig. 6. Analysis of the role of CD16 in $\gamma_1(\text{RGD})_3$ inhibition of NK cell cytotoxic activity. (a) Pretreatment of effector cells with 3G8 ($\gamma_1\text{k}$), a murine monoclonal antibody to CD16, reverses the inhibitory effect of $\gamma_1(\text{RGD})_3$. (b) $\gamma_1(\text{RGD})_3$ inhibits the lytic activity of CD16^+ , but not CD16^- NK clones. NK clones (Bacchetta *et al.*, 1993) were incubated with K-562 cells in the presence of $\gamma_1(\text{RGD})_3$ (100 $\mu\text{g/ml}$) as described in the legend to Figure 3. Expression of CD16 was confirmed at the time of the NK assay by fluorescence-activated cell sorting with monoclonal antibody 3G8. (c) Fab and F(ab')_2 fragments of $\gamma_1(\text{RGD})_3$ do not inhibit NK cell cytotoxic activity. The intact antibody (100 $\mu\text{g/ml}$) and Fab (papain digest) and F(ab')_2 (pepsin digest) fragments (300 $\mu\text{g/ml}$) were preincubated with target cells for 1 h at room temperature to favor binding, and then effector cells were added. $\gamma_1\text{NANP}$ and its fragments (open bars) served as controls. Fragments *per se* did not increase the spontaneous release of ^{51}Cr by target cells. Hatched bars denote the presence of $\gamma_1(\text{RGD})_3$ or its fragments. NK cell cytotoxic activity in the absence of inhibitor is denoted by the black bars. Results are expressed as percent lysis $\pm$ SD.

contrast to the restricted and specific recognition of CTLs. It is worth noting that RGD or similar hydrophilic motifs (e.g. GRD, RDG, RGE, DGR and GRGD) are present on human CD56 (Barton *et al.*, 1988; Hemperly *et al.*, 1990) and its murine counterpart, NK 1.1 (Yokoyama *et al.*, 1989). However, this does not rule out the involvement of other surface molecules expressing RGD like a 27 kDa molecule recently cloned from activated NK cells and encoded by a polymorphic gene (Dahl *et al.*, 1992). These proteins could

Fig. 7. $\gamma_1(\text{RGD})_3$ inhibits NK, LAK and ADCC-mediated cytotoxic activity, but not lysis of target cells mediated by MHC-restricted CD8⁺ and CD4⁺ CTLs. Inhibitors were $\gamma_1(\text{RGD})_3$ (■) or $\gamma_1\text{NANP}$ (▲). (a) Inhibition of NK cell cytotoxic activity. Dose response of the inhibitory effect of the lysis of K-562 cells by nonadherent PBLs from a healthy donor. The inset shows the percentage lysis in the absence of AgAb. (b) Inhibition of LAK cell-mediated cytotoxic activity. Dose response of the inhibitory effect of the lysis of non-small-cell lung carcinoma (TV9) cells by PBLs activated in the presence of 500 U/ml of rIL-2 for 6 days. The inset shows the percentage lysis in the absence of AgAb. (c) Inhibition of ADCC-mediated cytotoxic activity. Dose response of the inhibitory effect on the lysis of L₂C guinea pig leukemia cells preincubated with a chimeric (mouse/human) antibody to the L₂C idiotype by PBLs from a healthy volunteer. The inset shows the percentage lysis in the absence of AgAb. (d) Inhibition of lysis of EBV-transformed human B lymphocytes mediated by a CD8⁺ CTL clone. The CTL clone is specific for the peptide epitope 120–130 (MQWNSTAFHQQT) of the pre-S2 region of the HBenvAg and is restricted by the B62 HLA allele. Target cells consist of B62⁺ EBV-transformed human B lymphocytes pulsed with the corresponding synthetic peptide. The inset shows the percentage lysis in the absence of AgAb. (e) Inhibition of lysis of EBV-transformed human B lymphocytes mediated by a CD4⁺ CTL clone. The CTL clone is specific for the peptide epitope 193–207 (FFLLTRILTIPQSLD) of the pre-S2 region and is restricted by the DR2 HLA allele. Target cells consist of B62⁺ EBV-transformed human B lymphocytes pulsed with the corresponding synthetic peptide. The inset shows the percentage lysis in the absence of AgAb.

mimic the function of the RGD motif of the AgAb (see following). On the other hand, lack of CD16 on CTLs may explain, at least in part, the difference between NK cells and CTLs.

Counter-inhibition by anti-CD16 antibody underlines another key aspect of our study: inhibition requires occupancy of CD16. Immobilization of the AgAb at the cell surface through the Fc receptor could favor the orientation of the engineered loops and the formation of a bridge between effector and target cells. A model of the possible molecular interaction between the NK cell, the AgAb and the target cell is depicted in Figure 8a. We hypothesize that target cells possess complementary acceptor molecules for the (RGD)₃ loops and that NK cells express a molecule containing an RGD motif. Indeed, anti-(RGD)₃ antibodies bind effector cells. In addition, the monosialoganglioside GM2, which is known to inhibit NK cell cytotoxic activity (Bergelson *et al.*, 1989; Grayson and Ladisch, 1992), selectively down-regulates the expression of CD56 on effector cells (P. del Guercio and M. Zanetti, manuscript in preparation). These data suggest that CD56 could be the molecule expressing an RGD motif on effector cells that participated in the cytotoxic activity of NK cells. On the other hand, we cannot rule out the possibility that cross-linking of CD16 and the acceptor molecule for (RGD)₃ on NK cells

may yield a negative signal, such as that observed in B cells with cross-linking of Fc γ R and membrane immunoglobulin (Phillips and Parker, 1983, 1984). Whether $\gamma_1(\text{RGD})_3$ inhibits by interfering with adhesion or with a recognition/activation event remains to be proved. Our evidence is that $\gamma_1(\text{RGD})_3$ does not decrease the number of E:T conjugates (data not shown), favoring the view that it may interfere with the recognition/activation event.

It is somewhat paradoxical that $\gamma_1(\text{RGD})_3$ inhibits rather than enhances the cytotoxic effects of NK cells through antibody-mediated cell cytotoxicity (ADCC). One possible reason for this is that the surface antigens recognized by antibodies in a conventional ADCC phenomenon and the acceptor molecule for the RGD motif are qualitatively and/or quantitatively different. Another possibility is that the AgAb hinders the interaction between CD16 and a counter-receptor (ligand) on target cells (Figure 8b) when the (RGD)₃ loops engage with their acceptor, e.g. VLA-5 ($\alpha 5$, $\beta 1$), the fibronectin receptor present on K-562 cells that functions through RGD (Hemler, 1990; Hemler *et al.*, 1987). However, other molecules could be involved, as $\gamma_1(\text{RGD})_3$ inhibits killing of cells that lack the $\alpha 5$ subunit of VLA-5 (data not shown). That CD16[−] NK cell clones also lyse target cells reflects alternative activation via CD2 (Perussia, 1991) and bears no consequences for the model proposed

Fig. 8. Hypothetical model of the molecular interactions occurring during $\gamma_1(\text{RGD})_3$ -mediated inhibition of NK cell cytotoxic activity. (a) The model shows that $\text{A}^\text{B}\text{Ab}$ binds with the RGD-containing loops to acceptor molecules on the surface of target cells while the Fc portion of the molecule binds to CD16 on effector cells. The model hypothesizes the existence of a molecule on NK cells that expresses an RGD motif mimicking structurally and functionally the $\text{A}^\text{B}\text{Ab}$ loops. (b) Binding of the Fc portion of the $\text{A}^\text{B}\text{Ab}$ with the RGD motif hinders a complementary unidentified ligand structure expressed at the surface of target cells indicated by a protruding knob at the cell surface.

as these clones may have derived from fetal liver precursors and are representative of a minor (<1%) component of adult peripheral NK cells (Bacchetta *et al.*, 1993).

Our finding clearly shows the importance of spatial conformation in the biological function(s) of peptide ligands. Although monoclonal antibodies directed against several antigens expressed by NK cells have been shown to inhibit NK function (Dongworth *et al.*, 1985; Axberg *et al.*, 1987; Makgoba *et al.*, 1988), a putative NK receptor has not been isolated, and the molecular structure(s) that may account for the productive formation of NK cell–target cell conjugates remains unknown. By demonstrating the involvement of the RGD motif in NK cell cytotoxic activity, we have identified the first structural motif involved in how NK cells may recognize and lyse target cells. A molecule like $\gamma_1(\text{RGD})_3$ could be beneficial during bone marrow transplantation. NK cells are involved in the rejection of bone marrow cells in mice (Ghayur *et al.*, 1987; Bix *et al.*, 1991; Liao *et al.*, 1991) and possibly in humans (Lopez *et al.*, 1980; Drobyski *et al.*, 1990; Fischer *et al.*, 1990; Robertson and Ritz, 1990) although no clear correlation between NK cell activity and graft failure has been observed in patients with severe combined immunodeficiency (SCID; Buckley *et al.*, 1986). Thus, blockage of NK cells may be an important maneuver to favor engraftment of bone marrow transplants.

Implications for peptide folding

Several structural considerations can be made. First, antigenization of antibody is an efficient approach for stabilizing the conformation of the RGD peptide outside its natural environment. By grafting this peptide motif into an

antibody CDR loop, we succeeded in imparting a ligand property to the immunoglobulin molecule. Thus, antigenization of antibody is a new, efficient way to provide three-dimensional conformation and relative stability to oligopeptides, including RGD-based peptides. Interestingly, in fibronectin (Main *et al.*, 1992) and tenascin (Leahy *et al.*, 1992) RGD is also expressed at the tip of a β -loop of a domain with a topology similar to that of a constant (C) domain of an immunoglobulin and the second domain of human CD4. However, no amino acid similarity exists between these two groups of proteins. Thus, it should not be surprising that an RGD motif expressed within the molecular environment of an antibody CDR loop was not only exposed on the surface of the molecule but also biologically functional. We used the same approach to constrain conformationally and express, in an immunologically accessible way, short peptides from the human CD4 receptor molecule (Lanza *et al.*, 1993), a member of the immunoglobulin gene superfamily (Ryu *et al.*, 1990; Wang *et al.*, 1990). Whether the process of antibody antigenization is more efficient for peptides originating from proteins with an immunoglobulin-like fold awaits further experimental demonstration.

Antibody β -loops are, to some extent, independent of the physicochemical constraints that maintain the packing of the β -sheets in a conserved immunoglobulin framework (Chothia *et al.*, 1985). The molecular models shown in this study (Figure 3) helped to establish a structure–function correlate for the CDR3 loop constraining the foreign peptide. Our conformational exploration analyses indicate that RGD motif-containing loops exist in a limited number of metastable conformations, and that the conformationally constrained RGD motif has limited flexibility. Several factors probably contribute to this effect: the compactness of the rest of the protein sufficient to prevent the engineered loop from folding back on to the protein surface, the hydrophilic and highly charged nature of the engineered loops, and their covalent attachment at the end points. Indeed, engineered loops with the lowest energy conformation had very similar structure and spatial orientation (data not shown). In light of the fact that amino acids of surface loops tend to be more hydrophilic than those of the interior of the protein (Rose *et al.*, 1985), future determinations of possible loop conformation need to include solvent molecules in the calculations. However, even at the present stage of structure resolution, $\text{A}^\text{B}\text{Ab } \gamma_1(\text{RGD})_3$ can be a valuable tool for immunoprecipitating and identifying the acceptor molecules on target cells.

Antigenized antibody expressing $(\text{RGD})_3$ provided insights into the structural requirements for and specificity of the cytotoxic activity of NK cells. Solvent accessibility of the engineered β -loop and acquisition of a dipole characteristic could account for the biological activity of $\gamma_1(\text{RGD})_3$. Whether this functional motif on effector cells is sequence dependent, charge dependent or both is unclear. Electrostatic effects can play an important role because they are long-range forces. Nuclear magnetic resonance studies show that proteins that contain integrin-binding RGD sequences, such as kistrin, echistatin and the tenth type III module of fibronectin, express RGD at the tip of a β -turn (Adler *et al.*, 1991; Saudek *et al.*, 1991; Main *et al.*, 1992). The ligand function of RGD in different molecules varies depending on the surrounding residues and the overall molecular environment (Ruoslahti and Pierschbacher, 1987). It is important to recognize that a conformational RGD motif

can be mimicked by non-RGD sequences (Ruoslahti and Pierschbacher, 1987) and that synthetic RGD peptides can block adhesion interactions of non-RGD ligands on the basis of thermodynamic mimicry (Blobel *et al.*, 1992). RGD can have a wide range of ligand functions for various acceptor structures. Interestingly, the fusion motif (Thr-Asp-Glu) involved in sperm-egg fusion is expressed at the tip of a loop where it adopts a particular tertiary structure that creates an electrostatic effect neutralized by a large molar excess of RGD-containing peptides (Bronson and Fusi, 1990).

Materials and methods

Nonadherent peripheral blood lymphocytes

PBLs were isolated from heparinized blood of healthy donors by using a Ficoll-Hypaque (Histopaque 1077, Sigma) density gradient, washed, resuspended in RPMI 1640 and incubated for 2 h at 37°C to deplete adherent mononuclear cells. Nonadherent cells were harvested, washed and resuspended in RPMI 1640.

Continuous cell lines, T-cell and NK cell clones

Human erythroleukemia K-562 cells were obtained from ATCC and human non-small-cell lung carcinoma TV9 cells from Dr Nissi Varki (UCSD, Cancer Center). Cells were grown in RPMI 1640 supplemented with 4 mM glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 10 mM HEPES and 10% fetal calf serum (FCS) and harvested by centrifugation. CD4⁺ and CD8⁺ T-cell clones specific for hepatitis B envelope antigen (HBenvAg) were isolated and maintained in culture as previously described (Franco *et al.*, 1992) by using interleukin 2 (IL-2) and periodical restimulations with phytohemagglutinin (PHA) and allogeneic antigen-presenting cells (APC). Briefly, PBLs were plated (10⁵/well) in 96-well flat-bottomed plates in the presence of HBenvAg (10 µg/ml), and recombinant IL-2 (rIL-2) (40 U/ml) was added 5 days later. After an additional culture period of 5 days, growing cultures were expanded with rIL-2 and restimulated with antigen and autologous irradiated PBLs every 15 days. Cultures chosen for their capacity to proliferate in response to HBenvAg were cloned by limiting dilution at 0.3 cells/well with 1 µg/ml PHA, (Wellcome, Dartford, UK) rIL-2 and allogeneic APC as described (Barnaba *et al.*, 1989, 1990; Franco *et al.*, 1992). The CD8⁺ T-cell clone is specific for the peptide epitope 120–130 (MQWNSTAFHQT) of the pre-S2 region of the HBenvAg and is restricted by the B62 HLA allele. The CD4⁺ T-cell clone is specific for the peptide epitope 193–207 (FFLLTRILTIQSLD) and is restricted by the DR2 HLA allele. CD56⁺ NK cell clones were prepared from PBLs of a SCID patient successfully treated with fetal liver stem cell transplantation (Bacchetta *et al.*, 1993). They were isolated by cell sorting and expanded with a feeder cell mixture consisting of irradiated allogeneic PBLs (10⁶/ml) and Epstein-Barr virus (EBV)-transformed lymphoblastoid B cell lines (10⁶/ml), PHA (0.1 µg/ml) and rIL-2 (20 U/ml). After 10 days, CD16⁺ CD56⁺ CD3[−] cells were sorted a second time to eliminate contaminating T cells and cloned by limiting dilution. All NK clones were CD3[−] and CD56⁺. Expression of CD16 varied from negative to weakly positive. EBV-transformed B62⁺ and DR2⁺ lymphoblastoid B cells were prepared by EBV infection of PBL from a B62⁺ and a DR2⁺ donor (Barnaba *et al.*, 1990; Bacchetta *et al.*, 1993).

Synthetic peptides

Synthetic peptides RGDS, GRGDSPC and GdRGDSP were purchased from Telios Pharmaceuticals (San Diego, CA).

Monoclonal and polyclonal antibodies

Monoclonal antibody 3G8 (γ2a, κ) specific for CD16 was kindly given by Dr J. Unkeless (Mount Sinai School of Medicine, New York). Rabbit anti-Id antibodies were prepared against γ₁(RGD)₃ and made specific for the (RGD)₃ loop by extensive adsorption on insolubilized human IgG and murine λ light chains (F. Rossi, R. Billedda, Z. Ruggeri and M. Zanetti, manuscript in preparation). Anti-Id antibodies were purified using affinity chromatography with the synthetic peptide GRGDSPC insolubilized on epoxy-Sepharose 6B (Pharmacia-LKB). Their specificity was assessed using an enzyme-linked immunosorbent assay (ELISA). They bound to γ₁(RGD)₃, but not to other A₈Ab of the same isotype expressing unrelated peptides in CDR3 (data not shown).

Antigenized antibodies

For both A₈Ab, the D region of the parental variable H chain (V_H) gene (KAYSHG; residues 93–98) was mutagenized (Sollazzo *et al.*, 1989) to

introduce a single *KpnI*/Asp718 site to yield the intermediate sequence KVPYSHG (residues 93–99). The amino acid A94 was deleted and was replaced with the VP doublet encoded by the nucleotide sequence of the Asp718 cloning site. Subsequently, complementary oligonucleotides coding for one or three RGD copies were introduced between V94 and P95 of the mutagenized V_H region. The engineered V_HRGD and V_H(RGD)₃ encoded by the 2.3 kb *EcoRI* fragments were cloned upstream of a human γ₁ constant (C) region gene contained in the 12.8 kb vector pNγ₁ (Sollazzo *et al.*, 1989). Thirty micrograms of each DNA construct, pNγ₁RGD and pNγ₁(RGD)₃, was electroporated in the murine J558L cell line (2 × 10⁷ cells) by using a field strength of 750 V/cm. Transfected cells were cultured in Dulbecco's modified minimal Eagle's medium (DMEM) supplemented as previously described and then selected in the presence of neomycin (1.2 mg/ml) (G418; Gibco-BRL). G418-resistant clones secreting high levels of the A₈Ab were detected by screening supernatants in an ELISA, with horseradish peroxidase-conjugated goat antibodies to human immunoglobulin (Sigma) as probe. A₈Ab were purified from culture supernatants on protein A affinity columns. Fab and F(ab')₂ fragments were prepared by digestion with papain or pepsin by using immobilized enzymes (Pierce, Rockford, IL). The working conditions were established in pilot experiments. Digestion was carried out at 37°C for 4 h in sodium acetate buffer, pH 4.5 (for pepsin) or for 18 h in phosphate buffer, pH 7.2, containing cysteine-HCl (0.35%) (for papain). Undigested immunoglobulin molecules and Fc fragments were removed by chromatography on protein A-agarose columns. The purity of the digested material was checked on 12.5% and 8% SDS-polyacrylamide gels with or without β-mercaptoethanol.

NK cell cytotoxic assay

NK cell cytotoxic activity was determined by using a 4 h ⁵¹Cr release assay. Briefly, K-562 cells (target cells) were labelled with Na⁵¹CrO₄ (150 µCi/1 × 10⁶ cells) for 1 h at 37°C in an atmosphere of 5% CO₂ and then washed and suspended in culture medium supplemented with 10% FCS. One hundred microliters of ⁵¹Cr-labelled target cells (2.5 × 10⁵/ml) was mixed with 100 µl of nonadherent PBLs (effector cells) at an E:T ratio of 50:1 unless otherwise specified. The plates were incubated for 4 h at 37°C in 5% CO₂ and then centrifuged at 500 g for 4 min. One hundred microliters of supernatant was removed from each well and counted in a gamma counter. Spontaneous and maximal ⁵¹Cr release were determined by incubating target cells in medium alone or in the presence of 1% Triton X-100, respectively. The cytotoxic activity was calculated from triplicate wells as follows: [(experimental release – spontaneous release)/(maximal release – spontaneous release)] × 100. Maximal release was at least five times greater than spontaneous release. γ₁(RGD)₃ or γ₁NANP was added to the E:T suspension at a final concentration of 1–200 µg/ml. In some experiments, effector cells were preincubated with rabbit anti-Id antibodies (1–50 µg/ml) for 1 h at 4°C, and then target cells were added and the assay was continued as described before. In experiments in which Fab and F(ab')₂ fragments of γ₁(RGD)₃ (300 µg/ml) were used, target cells were similarly preincubated for 1 h at 4°C, then effector cells were added and the assay was continued as described before.

Lymphokine-activated killer (LAK) cell assay

LAK cell cytotoxic activity was determined by incubating ⁵¹Cr-labelled TV9 cells (target cells) with PBLs (effector cells) cultured for 6 days in the presence of 500 U/ml of IL-2, at an E:T ratio of 50:1, as described earlier. TV9 cells were chosen because they are not lysed by freshly prepared NK cells (<10% lysis; data not shown). The percentage of cytotoxicity was calculated as described before. Maximal release of chromium was at least five times greater than spontaneous release. γ₁(RGD)₃ or γ₁NANP was added to the E:T suspension at a final concentration of 50 µg/ml.

Antibody-dependent cytotoxic cells (ADCC) assay

ADCC were assayed as previously described (Dearman *et al.*, 1988). Briefly, the ADCC-mediating antibody used in the assay was a chimeric antibody consisting of a Fab from a mouse monoclonal anti-Id antibody to the L₂C idiotype antibody (Stevenson *et al.*, 1993) chemically linked to a double Fc of a human IgG1 molecule. This molecule is specific, non-modulating and very efficient in mediating ADCC by human effector cells. Control chimeric antibody was specific for a private idiotype determinant of tumor cell IgM from a patient with lymphoma. Human PBLs served as effector cells and were prepared as described earlier. L₂C guinea pig leukemia cells (target cells) were freshly prepared from the blood of tumor-bearing guinea pigs. They were purified using Ficoll-Hypaque and were then washed and labelled with ⁵¹Cr. After washing, labelled cells were resuspended at 2 × 10⁵/ml in medium containing 10% FCS. Then 50 µl of cell suspension was mixed with 50 µl of chimeric antibody or control antibody (10 µg/ml) and incubated for 15 min at 4°C. The dose of chimeric antibody added was determined in pilot experiments to ensure a plateau level of cell lysis.

Tests were done at an E:T ratio of 50:1 in triplicate wells of 96-well round-bottomed microtiter plates. γ_1 (RGD)₃ or γ_1 NANP was added to the E:T suspension at a final concentration of 50 μ g/ml. The plate was gently centrifuged (100 g for 3 min) to promote E:T contact. Finally, the plates were cultured for 4 h at 37°C and then centrifuged at 200 g for 5 min. The percentage of specific lysis was calculated as described.

CTL activity

CTL cytotoxic activity was determined by using a 4 h ⁵¹Cr release assay. ⁵¹Cr-labelled EBV-transformed lymphoblastoid cell clones (target cells) pulsed with synthetic peptide (FLLLRILTIQSLD for CD4⁺ and MQWNSTAFHQ for CD8⁺ clones) (4 μ g/ml for 4 h at 37°C) were mixed with CTL clones (effector cells) at an E:T ratio of 10:1 unless specified. The percentage of cytotoxicity was calculated as described before. Maximal release of chromium was at least five times greater than spontaneous release. γ_1 (RGD)₃ or γ_1 NANP was added to the E:T suspension at a final concentration of 1–50 μ g/ml.

Hydrophilicity analysis

Plots were generated using the Kyte–Doolittle algorithm (Kyte and Doolittle, 1982) and the sequence analysis program Mac Vector™ 3.5. The window size used in this analysis was 3. The hydrophathy values of each sequence of three amino acids are summed and then divided by the same window's size to obtain the average hydrophilicity per residue for that window. Values above the axis denote hydrophilic regions that are exposed on the outside of the molecule. Values below the axis indicate hydrophobic regions that tend to be buried inside the molecule or inside other hydrophobic regions. The Kyte–Doolittle scale was originally used for hydrophobicity profiles. The program Mac Vector™ 3.5 has reversed the signs of the values so that hydrophilicity is plotted instead.

Computer modelling methods

The coordinates of RGD and RGD₃ were built by using the program Homology (Biosym Technologies, San Diego, CA). Alignments of the sequences of the H and L chains of reference proteins with known coordinates were made by using the scheme of Kabat and Wu (Kabat *et al.*, 1987). The coordinates for the backbone atoms of amino acid residues in the conserved regions were obtained from two reference structures. Those for the H chain came from the mouse Fab fragment J539 (Suh *et al.*, 1986), and those for the L chain came from the antihemagglutinin 17/9 (Rini *et al.*, 1992). In the conserved region, when the amino acid type of the reference protein and the model matched, the coordinates for the sidechains were copied directly. When they differed, the sidechain was built from a standard amino acid template library. Bond lengths and angles were kept at their standard values. For the χ angles, the conformations of five other reference structures were examined. A conformation was chosen that was compatible with the majority of the other proteins. Loop coordinates also came from reference immunoglobulin structures; the protocol of Chothia and Lesk (1989) was used. The coordinates for the RGD and (RGD)₃ loops were generated using the method of Jones and Thirup (1986), wherein an α -carbon distance matrix was constructed for peptide segments on either side of the loop. The matrix was compared with those made from proteins of high resolution found in the Brookhaven Protein Data Bank (Bernstein *et al.*, 1977). Peptide segments (loops) were selected for which the root mean square (RMS) differences of the distance matrices were lowest and the number of residues in the loop were the same. Ten such loops were obtained and, for each model, one that had the least steric overlap with the rest of the protein, as judged by eye, was chosen. The final conformation for the loop in each model was determined through a combination of energy minimization and molecular dynamics by using the program Discover (Biosym Technologies). All atoms of the proteins except those of the loops were held fixed. Preliminary energy minimization was done to relieve the greater part of the strain inherent in the building procedure. Then molecular dynamics was done at a system temperature of 900 K. The trajectory was sampled at 1 ps intervals, and each snapshot was energy-minimized by using the steepest descents method. For RGD, the molecular dynamics was carried out for 100 ps; for (RGD)₃, the run was 20 ps long. For each model, the conformation studied was that with the lowest energy. Electrostatic potential energy contours were generated using the program DelPhi (Biosym Technologies; Gilson and Honig, 1988). The focusing technique was used, i.e. a preliminary calculation was done and the resulting grid of potential energy values was used as input to the final calculation, thus reducing boundary edge effects. The final grid was a cube, with sides measuring 15 Å, centered on the engineered loop.

Acknowledgements

This work was supported by a grant from the Council for Tobacco Research and NIH grant HD25787 (to M.Z.) and by I.S.S. Progetto di Ricerca

sull AIDS 7204-06 (to V.B.). We thank Drs E. Golub, C. MacLeod and Z. Ruggeri for suggestions and discussion of the work.

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Received on July 8, 1993