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Immunology

Synthetic peptide ligands of the antigen binding receptor induce programmed cell death in a human B-cell lymphoma

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ABSTRACT **Peptide ligands for the antigen binding site of the surface immunoglobulin receptor of a human B-cell lymphoma cell line were identified with the use of filamentous phage libraries displaying random 8- and 12-amino acid peptides. Corresponding synthetic peptides bound specifically to the antigen binding site of this immunoglobulin receptor and blocked the binding of an anti-idiotype antibody. The ligands, when conjugated to form dimers or tetramers, induced cell death by apoptosis *in vitro* with an IC_{50} between 40 and 200 nM. This effect was associated with specific stimulation of intracellular protein tyrosine phosphorylation.**

In an effort to develop a model for peptide-based therapy for human malignancies, we chose the antigen binding site of the surface immunoglobulin receptor of B-cell lymphomas as a target for peptides. The immunoglobulin receptor is tumor specific and unique to each patient. Antibodies raised against the immunoglobulin receptor idiotype (anti-Id) can arrest the growth of lymphoma cells *in vitro* (1) and can induce complete tumor regression *in vivo* (2, 3). However, the clinical utility of monoclonal anti-Id antibodies has been limited primarily by the difficulty in producing custom antibodies for each patient.

The antitumor effect of anti-Id antibodies observed in patients might be dependent on host effector systems, such as complement or antibody-dependent cellular cytotoxicity. Recent data, however, suggest that the direct antiproliferative effect exerted by the anti-Id antibodies, ultimately leading to programmed cell death, correlates best with clinical responses observed in patients (4). The direct antiproliferative effect observed is dependent on immunoglobulin cross-linking, which leads to transmembrane signaling through inositol phospholipid hydrolysis, protein kinase C activation, and tyrosine phosphorylation (5–7). Thus, the immunoglobulin receptor could be targeted with peptides that bind specifically to the receptor as surrogate ligands. The peptides themselves, when made multimeric, might inhibit growth by cross-linking immunoglobulin receptors or, if not, could be conjugated to deliver toxins or radionuclides. The tissue penetration of peptides, the ease of synthesis, and the ability to modify peptides are superior to antibodies. Finally, peptides tend to be less immunogenic than monoclonal antibodies.

Phage libraries can be generated to display millions to billions of random peptides on the minor coat protein pIII of the bacteriophage fd-tet (8–10). Such libraries can be used to find ligands or surrogate ligands to receptors. We describe in this paper the peptide ligands found by screening phage display libraries for binding to the purified IgM λ receptor of the human Burkitt lymphoma cell line SUP-B8 (11, 12). We evaluate their cytotoxic activity and their effect on signal transduction and cell death *in vitro*.

MATERIALS AND METHODS

Cell Lines and Immunoglobulin Receptor Purification. The B-cell lymphoma cell lines SUP-B8 (12), SU-DHL4 (13), and OCI-Ly8 (14) were cultured as published. A mouse monoclonal anti-Id antibody to the SUP-B8 immunoglobulin was produced (12). The *Escherichia coli* bacterial strains K91, MC 1061 F′, and DH5αF′ were used. SUP-B8 cells, SU-DHL4 cells, or tumor cells from patients were fused with the heterohybridoma K6H6/B5 and screened for production of the correct immunoglobulin isotype. The immunoglobulin was affinity purified (12, 15).

Affinity Selection Using Random Peptide Phage Libraries. Phage libraries displaying N-terminal random octapeptides linked to pIII with the spacers ASASA (single letter amino acid code) (library 1), or polyproline (mixture of P_4 and P_6) (library 2), or GG in the 12-mer library (library 3) were constructed (8). SUP-B8 immunoglobulin was immobilized on 96-well microtiter plates by adding 0.5 μg of immunoglobulin to each well. Plates were washed and blocked, and 10^{11} transducing units of bacteriophage from each library in 600 μl of Tris-buffered saline (50 mM Tris·HCl, pH 7.5/150 mM NaCl) were distributed equally among six wells and incubated for 2 hr at 4°C. The plates were washed with PBS. Bound phage were eluted with 0.1 M glycine HCl (pH 2.2), neutralized with 2 M Tris base, amplified in K91 cells, and precipitated for further rounds of panning. Individual phage clones were grown, purified, characterized by phage ELISA, and subjected to DNA sequencing (8).

Mutagenesis Phagemid Libraries. Three independent mutagenesis libraries were constructed like the random libraries (8) except that they were cloned into a pIII phagemid vector (pAFF2MBP). In this phagemid system, recombinant pIII expression was regulated by the inducible araB promoter and incorporated into phage particles along with wild-type pIII provided by helper phage (16–18). The oligonucleotides encoding the pIII inserts for the libraries were synthesized as follows. For each base addition, denoted in lowercase letters below, 70% were the "correct" base, encoding the amino acid of the input sequence, and the remaining 30% were split equally among the three remaining bases. If the consensus sequence had two amino acids at a position, the base composition was 40% each of the bases encoding the consensus (denoted as r = AC, s = GC, w = AT, y = TC) and 10% each of the two remaining bases. Positions with no preferred residue were left random, with the sequence NNK, where N = A, C, G, or T and K = G or T. The mutagenesis library oligonucleotides were synthesized (A library, 50% C TCT CAC TCC aag ccN tgg tgg gyN NNK agg gtN GGC GGC ACT GTT GAA AGT TGT, 50% C TCT CAC TCC aag ccN tgg tat gyN NNK agg gtN GGC GGC ACT GTT GAA AGT TGT; B library, C TCT CAC TCC ray tgg gsN rtg tgg NNK agg agg GGC GGC ACT GTT GAA AGT TGT; C library, C TCT CAC TCC tat NNK NNK gag gaN wtg tat agg agg GGC

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GGC ACT GTT GAA AGT TGT), gel purified, and enzymatically phosphorylated.

For each library 3 oligonucleotides were annealed with *Bst*XI-digested and purified vector in the following ratios: 1.6 pmol of pAFF2MBP/4.1 pmol of library oligonucleotide/4.1 pmol of GGAGTGAGAGTAGA/82 pmol of CTTTCAACAGT. The gapped plasmid was filled in by the addition of dNTPs and T7 DNA polymerase, precipitated, and resuspended. The DNA was electroporated into MC1061 F′ cells and grown in LB broth/ampicillin (100 μg/ml)/0.1% $MgSO_4$/0.25% KH_2PO_4-buffered medium containing 0.1% glucose for pIII repression. At early logarithmic phase, the cultures were infected with VCSm13 helper phage. Phagemid-producing cells were then induced and selected, respectively, by the addition of 0.02% arabinose and kanamycin (20 μg/ml). Phagemid were precipitated (8) and resuspended in PBS/0.1% bovine serum albumin. Each mutagenesis library was panned independently as described for the random libraries. Eluted phage were amplified by infection of DH5αF′ cells.

Phage ELISAs. Binding of phage to immobilized immunoglobulin was measured in an ELISA format (19). Competition ELISA was performed by adding monoclonal anti-Id antibody at various concentrations to the wells to compete with the binding of phage to SUP-B8 immunoglobulin.

Peptide Synthesis, Purification, and Polymerization. A peptide derived from one consensus sequence-containing clone from each mutagenesis experiment was synthesized and HPLC purified to >95% purity. The peptide content was confirmed by amino acid analysis, and the composition was verified by mass spectroscopy: peptide A, KPWWVSRVGGK–biotin; peptide B, DWAIWSKRGGK–biotin; peptide C, YSFEDLYRRGGK–biotin. All peptide monomers were synthesized with the C-terminal extension GGK–biotin, with the ε-amino group of lysine biotinylated. The following control peptides were synthesized: a peptide with the reverse amino acid sequence of one of the nonspecifically binding phage (control peptide), biotin–KSADWVRDYWS; a peptide with a randomly scrambled amino acid sequence of peptide C (scrambled peptide C), RDYSYERLFGGK–biotin; and a peptide with the reverse sequence of peptide C (reverse peptide C), biotin–KGGRRYLDEFSY. A tandem repeat of peptide C was synthesized with the amino acid sequence of peptide C incorporated twice, separated by 6 glycine residues (tandem repeat peptide C), YSFEDLYRRGGGGGGYSFEDLYRR. A control tandem repeat peptide was synthesized incorporating the scrambled sequence of peptide C (tandem repeat scrambled peptide C), RDYSYERLFGGGGGGRDYSYERLF. To form tetramers, equal volumes of 50 μM streptavidin and 200 μM biotinylated peptide monomer were mixed.

Peptide ELISAs. Biotinylated peptide monomer (0.4 μg per well) was immobilized on a microtiter well coated with 1 μg of streptavidin per well. SUP-B8 immunoglobulin or control immunoglobulin (0.25 μg) was added. Binding of immunoglobulin was detected with goat anti-human λ or κ/horseradish peroxidase conjugate. In the anti-Id peptide competition assay, 0.5 μg of SUP-B8 immunoglobulin per well was immobilized on a microtiter plate. Peptides were added at the concentrations shown. After 30 min, anti-Id antibody was added to each well and detected with goat anti-mouse IgG/horseradish peroxidase conjugate.

Proliferation Assays. Cells were plated in 96-well plates (5×10^3 cells per well) and incubated for 72 hr with peptides at the concentrations shown. Cell proliferation was measured by adding 1 μCi of [^{3}H]thymidine per well for the final 24 hr (1 Ci = 37 GBq). [^{3}H]Thymidine incorporation was measured by liquid scintillation counting of cell lysates harvested onto glass filters. All data shown are means of raw cpm.

Phosphotyrosine Assay. Cell lysates were electrotransferred from a reducing SDS/8% polyacrylamide gel to nitrocellulose and probed with the monoclonal anti-phosphotyrosine antibody 4G10 (4, 20).

Apoptosis Assay. SUP-B8 cells (2.5×10^6) were cultured with 2.5 μM peptides for 24 hr. Cells washed and resuspended in 50 μl of PBS were entrapped into 100 μl of melted 2% InCert agarose (FMC). The mixture was poured into a plug-forming mold. The gel plugs were treated overnight at 50°C with 100 mM EDTA, pH 8.0/1% Sarkosyl/1 mg of proteinase K per ml. The following day the plugs were treated for 2 hr at 50°C with RNase A, 0.1 mg/ml in 10 mM Tris·HCl/0.1 mM EDTA, pH 8.0. The gel plugs were inserted into a 1.5% agarose/TBE gel, electrophoresed, and stained with ethidium bromide.

All experiments shown in this paper were representative of at least three experiments with similar results.

RESULTS

Three different phage libraries, each containing $\approx 5 \times 10^8$ random 8-mer or 12-mer peptides, were subjected to four rounds of panning on the immunoglobulin of SUP-B8. Phage clones were purified. Their binding to the SUP-B8 immunoglobulin and to control immunoglobulins was tested in an ELISA format. Specificity was further tested by inhibiting the binding of the phage with a mouse monoclonal anti-Id antibody. After four rounds of panning, nearly all phage clones bound specifically. Their binding to the SUP-B8 immunoglobulin could in most cases be inhibited with anti-Id antibodies but not with anti-IgM or anti-λ light-chain antisera. Those phage clones that bound specifically to SUP-B8 were subjected to DNA sequencing. The deduced amino acid sequences of peptides from these phage are shown in Table 1. The peptide sequences fell into four groups of homology (groups A–D). Even though a consensus sequence could be determined for each of the four homology groups, some positions were poorly conserved, making it difficult to decide which peptide to synthesize for further studies.

To define the critical residues in each peptide family, three phagemid mutagenesis libraries were generated based on groups A, B, or C of Table 1. These libraries were constructed such that the oligonucleotides encoding the pIII insert were synthesized as a family of oligonucleotides based on the DNA sequence of the consensus peptides, varied to a defined degree. The predicted amino acid composition of the resultant libraries differed at each residue by ≈50% from the consensus sequence. The library size of each phagemid library was $\approx 10^8$ transducing units. After three rounds of affinity selection, phage clones binding to SUP-B8 immunoglobulin were identified and subjected to DNA sequencing. Table 2 summarizes the results, showing the sequences toward which the three libraries were biased (input) and the frequency of amino acids obtained after selection (output). Thus, the consensus sequences determined from screening random libraries were mostly confirmed by screening the mutagenesis libraries. Some exceptions were the emergence of I for V/M in the fourth position of mutagenesis library B, or K for R in the seventh position at the C terminus of the same group. Other poorly conserved positions from the random library screen were defined better, such as the sixth position of library A. Based on these results, a peptide from each mutagenesis consensus sequence was synthesized (peptides A–C).

The synthesized peptides A–C bound specifically to the SUP-B8 immunoglobulin and not to control immunoglobulin receptors from other B-cell lymphomas or to normal polyclonal IgM (Fig. 1). The control peptides with the scrambled or reverse amino acid sequence of peptide C did not bind to SUP-B8 immunoglobulin. Additionally, peptides A–C inhibited the binding of the bivalent anti-Id to SUP-B8 immunoglobulin (Fig. 2), while the control peptide did not. None of

Table 1. Amino acid sequences of ligands to Sup-B8 immunoglobulin

Group	Library	Peptide sequence
A	1	K P W Y V S R V
	2	K P W Y V G R P
	2	K P W W V T R V
	2	K P W W V S R V
	2	K P W W V V R L
	1	G K P W W A S R
	1	E K P W W A V R
	1	G K P W Y A G R
	2	G K P I W A G R
	1	K P S N V S R V
	1	Y Y C S P W C D
	1	V P W Y K Q S T
	1	A P W Y R V S P
	1	L P W Y L Y P S
	2	Q K P I W V T R
	3	K N G P W Y A Y T G R D
	Consensus	K P W * † X R V
B	2	D W A V W N R R
	2	N W A V W T K R
	2	N W G M W S K R
	Consensus	‡ W § ¶ W X ‖ R
C	2	Y V F E D L F R
	2	M P E D F Y R R
	3	F G I L T E E M Y R R W
	3	L R Y T Q E E M Y R R W
	3	H Y V H E D L Y R R V K
	3	V T G Y T M D V L Y R R
	Consensus	Y X X E D L R R R
D	2	S E P V D H G L
	2	V D P V D H G L
	2	V P I D H G T
	Consensus	X X P V D H G L

Amino acid sequences of phage clones that bound specifically to SUP-B8 immunoglobulin were grouped according to their homology (group A–D). The library from which each isolate was derived is shown in the second column. Library 1, 8-mer ASASA-pIII; library 2, 8-mer P_4-pIII and 8-mer P_6-pIII; library 3, 12-mer GG-pIII. For each of the homology groups, a consensus sequence is shown. *, W/Y; †, V/A; ‡, N/D; §, A/G; ¶, V/M; ‖, R/K.

the peptides inhibited the binding of anti-IgM or anti-λ antisera to SUP-B8 immunoglobulin (data not shown).

The peptide monomers themselves had no effect on the proliferation of SUP-B8 cells *in vitro* (data not shown). This was expected since it has been demonstrated that cross-linking of the surface immunoglobulin receptors is required to induce an antiproliferative effect in B-cell lymphoma cell lines (21). To cross-link surface immunoglobulin receptors, we made peptide polymers of biotinylated peptides bound to tetravalent streptavidin. The resulting putative peptide ligand tetramers were reproducibly cytotoxic, with an IC_{50} of 60–200 nM (Fig. 3*A*). After 3 days, only cell fragments were seen in the treated wells. The control peptide tetramer (Fig. 3*A*), scrambled peptide C tetramer (data not shown), reverse peptide C tetramer (data not shown), and streptavidin (data not shown) had no apparent effect on the cells. The peptide ligand tetramers had no influence on the proliferation of the control B-cell lymphoma cell lines OCI-Ly8 (Fig. 3*B*) or SU-DHL4 (data not shown), indicating that the cytotoxic effect was tumor specific.

A tandem repeat form of peptide C was synthesized as a 24-mer peptide incorporating the sequence of peptide C twice, separated by six glycine residues. This tandem repeat peptide C was as cytotoxic to SUP-B8 cells as the tetramers (Fig. 4*A*), with an IC_{50} of ≈40 nM. A control tandem repeat

Table 2. Affinity selection (panning) with three mutagenesis libraries

		Mutagenesis library A							
Input		K	P	W	W/Y	V/A	X	R	V
Output		K20	P19	W18	Y10	V18	S14	R18	V11
(*n* = 20)			S1	G1	W8	A2	T3	G2	L4
				Q1	F1		L1		I3
					S1		A1		A1
							Q1		G1
Consensus		K	P	W	W/Y	V	S	R	V
		Mutagenesis library B							
Input		N/D	W	A/G	V/M	W	X	R	R
Output		D23	W27	A16	I12	W26	S13	K27	R27
(*n* = 27)		N3		G10	V8	G1	N7		
		K1		P1	L7		M6		
							R1		
Consensus		D	W	A	I	W	S	K	R
		Mutagenesis library C							
Input	Y	X	X	E	D/E	L/M	Y	R	R
Output	Y24	S8	F14	E20	D20	L22	Y23	R19	R24
(*n* = 24)		T4	I4	Q3	E2	M2	F1	K5	
		V4	L4	G1	S1				
		F2	M1		A1				
		A2	Y1						
		Q,Y1							
		E,G1							
Consensus	Y	S	F	E	D	L	Y	R	R

Frequency of amino acids found at each position after three rounds of panning with libraries biased toward the input sequence. Consensus sequence of each mutagenesis experiment is shown. Total number of phage clones sequenced is shown in parentheses.

dimer with a randomly scrambled sequence of peptide C did not inhibit proliferation of SUP-B8 cells. The tandem repeat peptide C had no effect on proliferation of the control B-cell lymphoma cell lines OCI-Ly8 (Fig. 4*B*) or SU-DHL4 (data not shown). Furthermore, a peptide dimer made by allowing the oxidation of a peptide A variant synthesized with a C-terminal cysteine to form disulfide bonds was equally cytotoxic (data not shown).

The cytotoxic effect of the peptide ligand tetramers was reproducibly associated with specific phosphorylation of intracellular protein tyrosines. A pattern of tyrosine phosphorylation comparable with that seen with anti-IgM antibody (Fig. 5, lane 2) occurred within 1 min of exposure of SUP-B8 cells to peptide A tetramer (lanes 4–8). No increase in protein tyrosine phosphorylation was seen with the control peptide tetramer (lane 3) or with streptavidin alone (data not shown). Equal loading of all lanes in Fig. 5*A* was demon-

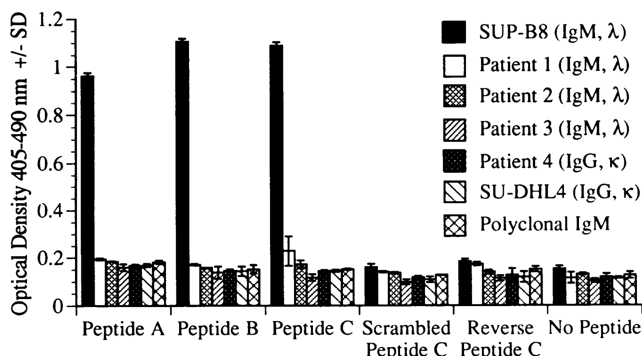

FIG. 1. Binding of purified SUP-B8 immunoglobulin or control immunoglobulins to biotinylated peptide monomers immobilized on a streptavidin-coated microtiter plate measured in an ELISA. Data shown are means of duplicate samples ± SD.

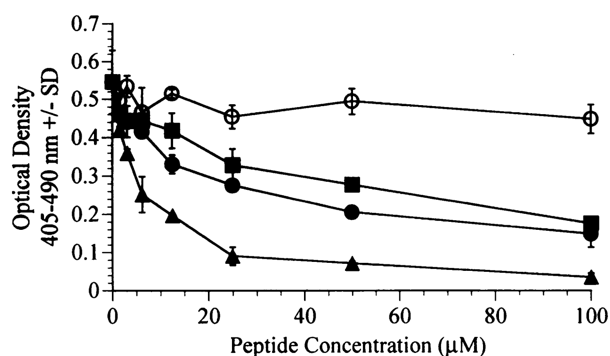

FIG. 2. Inhibition of anti-Id antibody binding to SUP-B8 immunoglobulin by increasing concentrations of peptide monomers measured in an ELISA. ●, Peptide A; ■, peptide B; ▲, peptide C; ○, control peptide. Data shown are means of duplicate samples ± SD.

strated in Fig. 5*B*, where a protein with a 76-kDa apparent molecular mass is detected in the same blot as Fig. 5*A* with a goat anti-mouse IgG antibody. There was no effect on protein tyrosine phosphorylation of the irrelevant OCI-Ly8 cells with peptide A tetramers (data not shown).

The SUP-B8 cells underwent programmed cell death when stimulated with the peptide tetramers. DNA fragmentation, a characteristic feature of cells undergoing apoptosis, was seen after 24 hr of incubation with peptide A–C tetramers, but not with the scrambled peptide C tetramer or reverse peptide C tetramer (Fig. 6). DNA fragmentation was not seen in the unrelated OCI-Ly8 cells incubated with peptide A–C tetramers or the control peptide (data not shown). Similarly, the tandem repeat peptide C and not the tandem repeat scrambled peptide C caused DNA fragmentation (data not shown).

DISCUSSION

These experiments demonstrate the application of phage display libraries in the discovery of surrogate ligands to a cell surface receptor. Previous studies have reported the use of peptide libraries in the identification of epitopes of immunoglobulins for which the specific antigen was known (8, 10, 24). In the example described here, several groups of surrogate ligands were found. Within each group, some amino acid residues were conserved, while others were not. Screening mutagenesis libraries helped to further define the critical residues. However, the consensus sequences derived from screening mutagenesis libraries were not greatly different from the consensus sequences obtained from the random library screen. Thus, it may not be necessary to screen mutagenesis libraries in future cases.

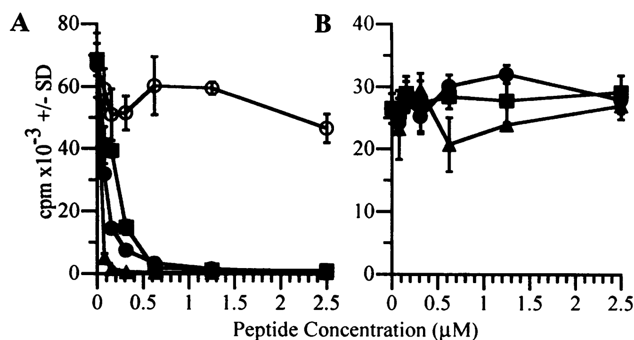

FIG. 3. Effect of peptide tetramers on cell proliferation measured by [^{3}H]thymidine incorporation. SUP-B8 cells (*A*) or OCI-Ly8 cells (*B*) were incubated with peptide tetramers at increasing concentrations for 72 hr. ●, Peptide A tetramer; ■, peptide B tetramer; ▲, peptide C tetramer; ○, control peptide tetramer. Data shown are means of raw cpm of quadruplicate samples ± SD.

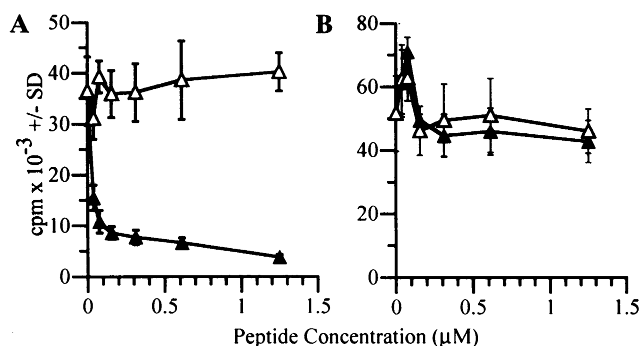

FIG. 4. Effect of increasing concentrations of tandem repeat peptide dimers on 72-hr SUP-B8 cell (*A*) or OCI-Ly8 cell (*B*) proliferation measured by [^{3}H]thymidine incorporation. ▲, Tandem repeat peptide C; △, tandem repeat scrambled peptide C. Data shown are means of raw cpm of quadruplicate samples ± SD.

It remains to be determined in what proportion of B-cell tumors surrogate ligands of the antigen binding receptor can be found. Short linear peptides, such as those described here, might fail to mimic antigens determined by discontinuous residues of proteins, by carbohydrates, lipids, or nucleic acids. Receptors on lymphomas may in fact be selected for binding to such complex structures (22). On the other hand, each binding site may have a shape that can be occupied by

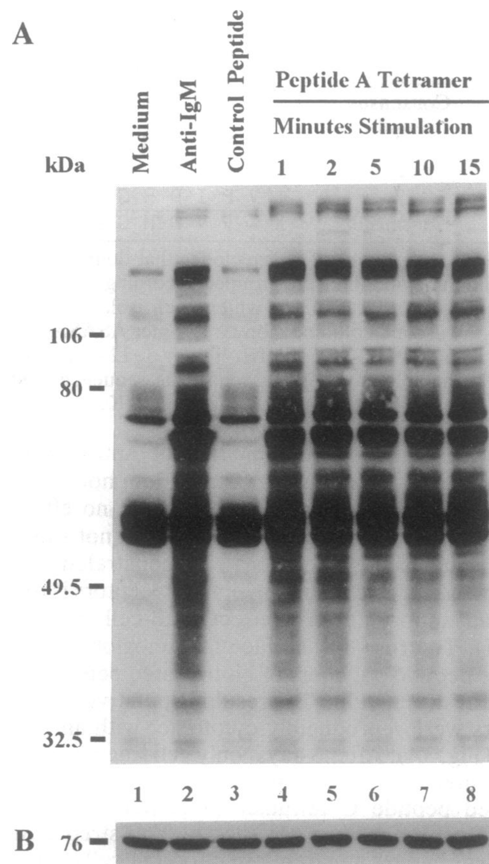

FIG. 5. Effect of peptide tetramers on signal transduction of SUP-B8 cells. Western blot showing intracellular protein tyrosine phosphorylation in SUP-B8 cells stimulated with peptide A tetramers. SUP-B8 cells were stimulated with medium (lane 1), anti-IgM polyclonal antiserum (lane 2), and 5 μM control peptide tetramer (lane 3) for 2 min or with 5 μM peptide A tetramer for 1, 2, 5, 10, or 15 min (lanes 4–8). (*B*) To show equal loading in all lanes in *A*, the blot was first stained with a goat anti-mouse IgG-biotin antibody followed by streptavidin horseradish peroxidase.

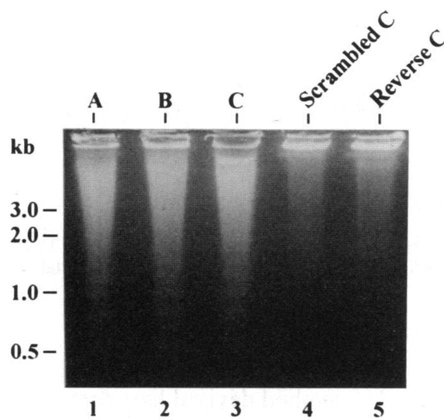

FIG. 6. DNA fragmentation in SUP-B8 cells incubated with peptide tetramers. DNA from SUP-B8 cells incubated for 24 hr with 2.5 μM peptide tetramers was electrophoresed on an agarose gel and stained with ethidium bromide. Lanes: 1, peptide A tetramer; 2, peptide B tetramer; 3, peptide C tetramer; 4, scrambled peptide C tetramer; 5, reverse peptide C tetramer.

a short peptide regardless of the true antigen for which the site was selected.

This previously undescribed specific cytotoxic activity of peptides, seen in the absence of effector cells or complement, may provide the basis for an additional form of therapy for lymphoma. The cytotoxic activity is mediated through the transmembrane immunoglobulin receptor as demonstrated by the rapid and specific increase in tyrosine phosphorylation after exposure to the peptide ligand tetramers, followed by apoptosis. We know that approaches targeting the immunoglobulin receptor can have major therapeutic effects in murine model systems and in human patients with B-cell lymphoma (2, 3, 15). Evidence of signal transduction induced by antibodies against the immunoglobulin receptor on human lymphomas, evaluated by the degree of tyrosine phosphorylation as was done in this study, predicts which patients will respond clinically to anti-idiotypic therapy (4). This provides a tool to evaluate the potential therapeutic benefit of surrogate peptide ligands *in vitro*. Several issues must be addressed in order to make peptides into therapeutic agents, such as their stability *in vivo* and their rapid clearance from blood. If needed, the incorporation of D-amino acids or other structural modifications of the peptide can be explored. Human lymphomas established in *scid* mice (23) provide a suitable *in vivo* model to test the therapeutic efficacy of synthetic peptide ligands. Indeed, we have now established conditions for growing the SUP-B8 tumor in *scid* mice and will now be able to determine whether peptides A–C or their derivatives can act as antilymphoma drugs.

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