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A recombinant immunotoxin that is active on prostate cancer cells and that is composed of the Fv region of monoclonal antibody PR1 and a truncated form of *Pseudomonas* exotoxin

(single-chain immunotoxin/chemotherapy/immunotherapy)

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ABSTRACT **Monoclonal antibody PR1 binds to the surface of normal prostate cells and to adenocarcinomas of the prostate. The cDNAs coding for the heavy and light chain variable regions of monoclonal antibody PR1 were cloned by PCR techniques. A recombinant toxin was then constructed that has the heavy chain variable region of monoclonal antibody PR1 connected to the light chain variable region by a flexible peptide linker to create a single-chain Fv; the Fv in turn is fused to a truncated form of *Pseudomonas* exotoxin. The resulting recombinant immunotoxin PR1(Fv)-PE38KDEL was produced in *Escherichia coli* and accumulated in inclusion bodies. After denaturation and renaturation, active monomeric molecules with a molecular mass of ≈65 kDa were purified to homogeneity. PR1(Fv)-PE38KDEL binds specifically to cells containing the PR1 antigen and is very cytotoxic toward a subset of LNCaP cells that express the PR1 antigen on their surface.**

PR1 is a recently isolated monoclonal antibody (mAb) that was obtained by immunizing BALB/c mice with a strain of a human prostate adenocarcinoma maintained in short-term culture (1). The mAb binds to an antigen that is present on the surface of human prostate cancer cells and on normal prostate tissue. By using immunohistochemical methods, mAb PR1 has been found to react with 19 of 20 samples of adenocarcinomas of the prostate (I.P., E. Lovelace, A. R. Rutherford, S.K., and D. Peehl, unpublished data). The PR1 antigen is not expressed on most normal human cell types but is present in low amounts in a few normal human tissues.

Immunotoxins composed of carcinoma-specific antibodies attached to bacterial or plant toxins are potent cytotoxic agents that can cause complete tumor regression in experimental animals (1–3). Such immunotoxins are best made using IgG; unfortunately, mAb PR1 is an IgM. The conjugation of IgM molecules to toxins is difficult and the resulting immunotoxins are obtained in low yields, are hard to purify, and may have low activity.

This laboratory has shown (for review, see ref. 1) that recombinant immunotoxins that are composed of the variable regions of the heavy and light chains (V_H and V_L, respectively) of antibodies that are connected to each other by a peptide linker and fused to a truncated form of *Pseudomonas* exotoxin (PE) are very active cytotoxic agents. They have the same or better potency than immunotoxins made by chemical conjugation methods. The production of single-chain immunotoxins in bacteria can circumvent the problems observed with the conjugation of IgM molecules to toxins. Here we describe the cloning of cDNAs encoding the V_H and V_L of mAb PR1. We also describe the construction and characterization of the single-chain immunotoxin PR1(Fv)-PE38KDEL that binds to and is selectively cytotoxic toward a prostate carcinoma cell line that expresses the PR1 antigen.

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MATERIAL AND METHODS

Cloning of cDNA Encoding V_L and V_H of mAb PR1 and Plasmid Constructions. DNA fragments encoding the V_H or V_L of mAb PR1 were obtained by PCR amplification of (random primed) reverse-transcribed PR1 hybridoma RNA as described (4). V_H cDNA was amplified with the primer pair PR1-H1 (5′-CAGTRDCTRMAGGTGTCCATATGGATGTGCAGCTGGTGGAGTCTGG-3′) and PR1-H2 (5′-GGAGACAAGCTTGAAGACATTTGGGAAGGACTGACTC-3′). V_L cDNA was amplified with PR1-L1 (5′-GTCTCCTCAGGCGGAGGGGGATCCGGTGGTGGCGGATCTGGAGGKGGCGGMAGCGAHRTTGTGATGACCCAGTCTCC-3′) and PR1-L2 (5′-AGTTGGTGCAGCATCAAAAGCTTTKAKYTCCAGCYTKGTGCC-3′). These primers contain *Nde* I, *Bam*HI, or *Hin*dIII sites (underlined) to facilitate cloning. The sequences of cloned PCR products were determined using a United States Biochemical Sequenase kit. To make a plasmid for expression of PR1(Fv)-PE38KDEL, the DNA fragments encoding PR1 V_L and V_H were combined by "PCR-splicing" techniques (4) to code for a single-chain Fv held together by a $(Gly_4\text{-}Ser)_3$ peptide linker. Amplification primers used for V_L were L1 and L2; for V_H we used H1 but replaced H2 (anneals to IgM heavy chain constant region C_H1) by H3 (5′-GGATCCCCCTCCGCCTGAGGAGACGGTGACTGAGGTTCCT-3′), which anneals to the 3′ end of the V_H sequence and codes for a part of the linker. The PCR fragment obtained after PCR-splicing (primers H1 and L2) of fragments from separate V_H and V_L reaction mixtures (primers H1 plus H3 and L1 plus L2) was cleaved with *Nde* I and *Hin*dIII to replace the B3(Fv) coding region of pULI9 [encoding B3(Fv)-PE38KDEL (with C3 connector, ref. 5)] to make pULI40 (see Fig. 3).

RNA Sequencing. Direct sequencing of RNA was performed essentially as described (6). mRNA (5 μg) and ^{32}P-labeled primer (10 ng; 5′-CATTTGGGAAGGACTGACTC-3′ complementary to the murine IgM C_H1 sequence) were incubated in 15 μl (250 mM KCl/10 mM Tris Cl, pH 8) for 3 min at 80°C followed by 1 hr at 45°C. This RNA-primer-annealing solution (3 μl) was then added to a mixture of 2.5 μl of chain elongation/termination mixture, 1.5 μl of 5× reverse transcriptase (RT) buffer, and 0.5 μl (5 units) of avian myeloblastosis virus (AMV) RT [elongation/termination mixture is all four dideoxynucleotide solutions from Seque-

Abbreviations: mAb, monoclonal antibody; V_H, V_L, and C_H, heavy and light chain variable regions, respectively, and heavy chain constant region; PE, *Pseudomonas* exotoxin; FACS, fluorescence-activated cell sorter.

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nase kit/AMV RT/5× RT buffer from BRL] and incubated for 1 hr at 50°C. Sequenase stop buffer (5 μl) was added, and the samples were boiled for 3 min and loaded on sequencing gels.

Expression of Recombinant Protein. PR1(Fv)-PE38KDEL was produced in *Escherichia coli* BL21 λDE3 (7) containing pULI40 and purified as described (8).

Immunofluorescence and Immunoperoxidase Studies. Binding of mAb PR1 and PR1(Fv)-PE38KDEL was detected by immunofluorescence as described (9), by using goat anti-mouse Fab IgG conjugated to rhodamine for PR1 IgM and rabbit anti-PE IgG and goat anti-rabbit IgG conjugated to rhodamine for PR1(Fv)-PE38KDEL. Immunoperoxidase localization of the antigen on frozen sections was performed as described (9).

Cytotoxicity Assays. Inhibition of protein synthesis was determined in 96-well plates (10). For competition of cytotoxicity, 15 μg of mAb PR1 or 13F5 was added 15 min prior to addition of toxin. Alternatively, the assays were combined with cell sorting with antibody-coated magnetic beads (see below).

Cell Sorting by a Fluorescence-Activated Cell Sorter (FACS) or with Antibody-Coated Magnetic Beads. PR1-positive LNCaP cells were enriched using a FACS (11). The original LNCaP sample had 1–3% PR1-positive cells whereas the sorted population contained up to 40% positive cells. Antigen-expressing cells were also separated from antigen-negative cells by means of antibody-coated magnetic beads (12). Beads for isolation of PR1-expressing cells were prepared by overnight incubation of anti-mouse IgM beads (Advanced Magnetics, Cambridge, MA) with mAb PR1 in phosphate-buffered saline (PBS)/0.1% bovine serum albumin at 4°C and subsequent washing (twice) in PBS/0.1% bovine serum albumin. Beads for sorting of OVCAR3 cells were prepared by coating the anti-mouse IgM beads with mAb 13F5 (recognizing an antigen on OVCAR3), by using the same conditions as for mAb PR1. Cells were grown, incubated with beads, and sorted in 25-cm² flasks (Costar 3025) as described (12) with the modification that the cells were washed by gentle pipetting about 20 times to dissolve conglomerates of live and dead cells when sorting samples from cytotoxicity assays.

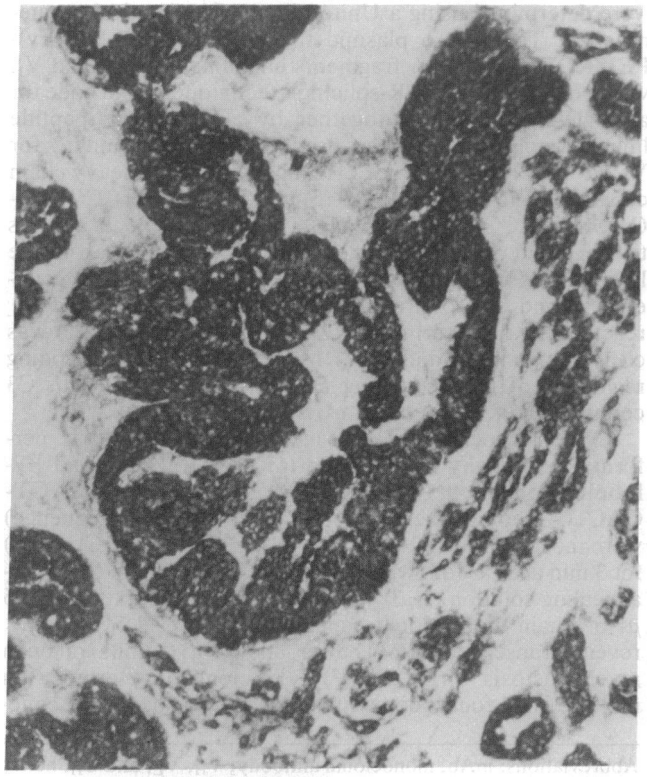

FIG. 1. Reactivity of mAb PR1 with adenocarcinoma of the prostate. Specific binding of tumor tissue is shown by immunoperoxidase staining of a frozen section (9).

RESULTS

mAb PR1 has been found to react with >90% of prostate carcinoma samples tested. A typical example of its reactivity with a human prostate cancer sample is shown in Fig. 1. One feature of mAb PR1 is that it appears to react with all the cancer cells in the sample (I.P., E. Lovelace, A. R. Rutherford, S.K., and D. Peehl, unpublished data). This property indicates the mAb may be useful for targeting prostate cancer cells with immunotoxins. To stably produce an agent capable of killing prostate cancer cells, PCR techniques were used to clone cDNAs encoding the variable regions of mAb PR1 and these cDNAs were used to make a single-chain immunotoxin PR1(Fv)-PE38KDEL.

Construction of a Plasmid for Expression of PR1(Fv)-PE38KDEL. Single-stranded DNA obtained by reverse transcription of hybridoma mRNA was used as a template. The PCR primers contained "consensus" sequences present at

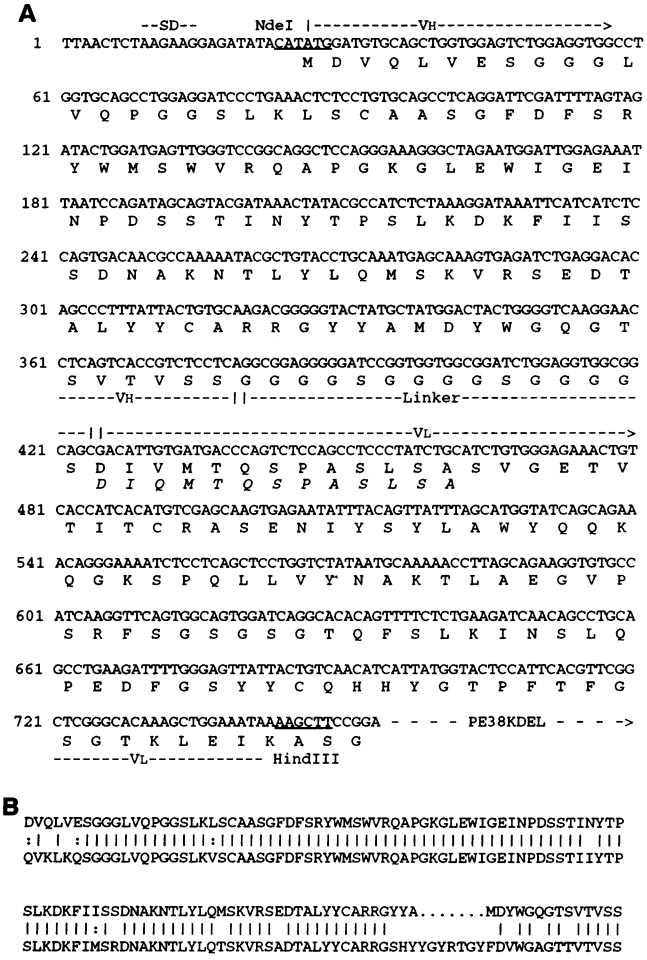

FIG. 2. Nucleotide sequence encoding the V_H and V_L regions of mAb PR1. (*A*) The V_H coding sequence extends from positions 29 to 375; V_L extends from positions 426 to 743. The deduced amino acid sequence is shown; the amino acid sequence determined by Edman sequencing of the V_L is in italic type. A difference at position 431 between the protein sequence of the PR1 κ chain and the cloned sequence is due to the primer L1 that was used for the PCR. The V_H sequence was confirmed by RNA sequencing. (*B*) Similarity between the V_H regions of mAb PR1 and mAb K1.

the beginning and end of genes for murine antibody variable domains or were complementary to the beginning of the IgM constant region (for cloning of V_H). The amplified variable region-encoding DNA fragments were linked together by PCR-splicing techniques so that the gene product of the resulting fusion gene is a single-chain Fv that has the V_H and V_L regions connected by a $(Gly_4\text{-}Ser)_3$ peptide linker (4). This Fv gene was inserted into a vector coding for a truncated derivative of PE (PE38KDEL), resulting in a plasmid for expression of the single-chain immunotoxin PR1(Fv)-PE38KDEL.

To confirm that the cloned cDNAs encoded the variable regions of mAb PR1, we obtained a partial sequence of the amino terminus of the light chain from purified mAb PR1 and found it to be NH_2-DIQMTQSPASLSA. This sequence was identical to the deduced protein sequence of the cloned V_L DNA (Fig. 2*A*), except for a Gln → Val exchange of the third amino acid, which was introduced by our consensus PCR primer. We could not obtain the amino-terminal sequence of the PR1 heavy chain, probably because its amino terminus was blocked (M. Lively, personal communication). Therefore, we directly sequenced RNA isolated from the PR1-producing hybridoma by using a ^{32}P-radiolabeled primer (complementary to IgM C_H1 coding sequences) to initiate polymerization. This technique excludes PCR-derived artifacts, such as selective amplification of only one of several DNA species. Only one sequence was obtained. Therefore, this represents the major species of heavy chain mRNA in mAb PR1-producing cells and it was identical to the sequence of cloned PR1(V_H). Sequence analyses of the cloned cDNAs showed the light chain of PR1 to be κ class V (13). It is almost identical to the light chain of the anti-idiotype mAb A25.9.7 (14). The V_H belongs to murine class 3B (13); it shows a remarkable similarity to mAb K1 with only one major difference in complementarity-determining region 3 (Fig. 2*B*). mAb K1 does not react with prostate cells but does react with many ovarian carcinomas, mesotheliomas, squamous cell carcinomas, and normal mesothelium (15–17).

Expression, Renaturation, and Purification of PR1(Fv)-PE38KDEL. *E. coli* BL21 (λDE3) cells harboring the plasmid pULI40 (Fig. 3*A*) for expression of PR1(Fv)-PE38KDEL accumulate recombinant immunotoxin upon isopropyl β-D-thiogalactoside induction in intracellular inclusion bodies. Those inclusion bodies were isolated, and the recombinant toxin was solubilized, reduced, and refolded to active immunotoxin by a rapid-dilution method containing redox-shuffling and aggregation-preventing additives in the refolding buffer (9). Properly folded molecules were purified to near homogeneity by ion-exchange and size-exclusion chromatography. The amount of recombinant protein in inclusion bodies, the purity of the inclusion bodies, and composition of the final product after refolding and purification are shown in Fig. 3*B*.

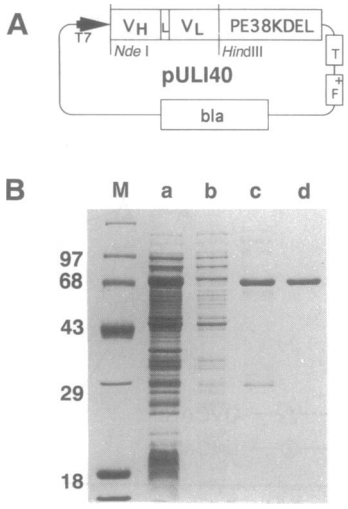

FIG. 3. Expression and purification of the recombinant immunotoxin PR1(Fv)-PE38KDEL. (*A*) Plasmid pULI40 for expression of PR1(Fv)-PE38KDEL. (*B*) Reducing SDS/PAGE on a Coomassie blue-stained 12.5% polyacrylamide gel. Lanes: a, total protein of cells producing PR1(Fv)-PE38KDEL; b, supernatant of sonicated cells; c, inclusion bodies; d, immunotoxin after renaturation, ion-exchange chromatography, and gel filtration; M, molecular mass standards are indicated on the left in kDa.

Specific Binding of PR1(Fv)-PE38KDEL to Antigen-Expressing Carcinoma Cells. mAb PR1 binds specifically to an antigen that is abundant on prostate cancers and normal prostate (I.P., E. Lovelace, A. R. Rutherford, S.K., and D. Peehl, unpublished data). However, the antigen is present in only a small proportion of cells from the cell line LNCaP and is not present on cell line DU145 (unpublished data). Because it was difficult to assess the cytotoxic action of PR1(Fv)-PE38KDEL on cells of which only 1–3% express the antigen, we used FACS to enrich for PR1-positive cells. After one cycle of sorting, 20–40% of the LNCaP cells were strongly PR1-positive, and these were used for immunofluorescence and cytotoxicity experiments described below.

The ability of PR1(Fv)-PE38KDEL to bind selectively to antigen-expressing LNCaP cells is shown in Fig. 4. The binding was specific because it was displaced by excess mAb PR1 at 50 μg/ml but not by a control IgM (mAb 13F5). The signals observed with the recombinant immunotoxin were weaker than observed with equivalent concentrations of PR1, probably in part due to the differences in binding affinity between the polyvalent (IgM) and the monovalent (Fv) molecules. Cell lines that did not bind mAb PR1 also gave no fluorescent signal with the recombinant immunotoxin (Table 1).

Cytotoxicity of PR1(Fv)-PE38KDEL Toward Various Carcinoma Cells. The toxicity of PR1(Fv)-PE38KDEL was ex-

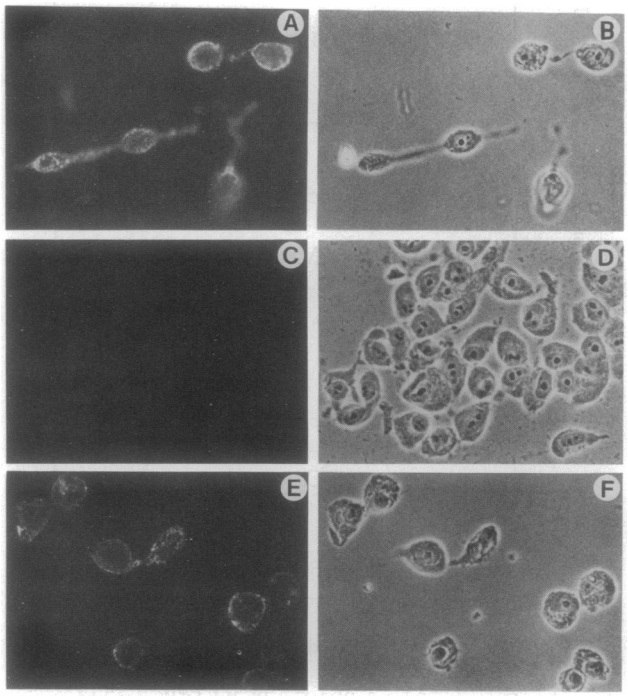

FIG. 4. Specific binding of PR1(Fv)-PE38KDEL to LNCaP cells enriched by using a FACS. Immunofluorescence of PR1(Fv)-PE38KDEL (10 μg/ml). (*A*) Without competition. (*C*) With mAb PR1 (50 μg/ml). (*E*) With control IgM (50 μg/ml). (*B*, *D*, and *F*) Phase-contrast pictures of the fields shown in *A*, *C*, and *E*, respectively. (×250.)

Table 1. Specificity and cytotoxicity of PR1(Fv)-PE38KDEL

Cell line	Carcinoma	PR1 binding	PR1(Fv)-PE38KDEL Binding	PR1(Fv)-PE38KDEL ID_{50} (ng/ml)
LNCaP	Prostate	+++ (het)	+ (het)	0.8 (250)
OVCAR3	Ovarian	−	−	>1000
KB	Epidermoid	−	ND	>1000

ID_{50} is the concentration of toxin that reduces protein synthesis of cells by 50%. het, Heterogenous immunofluorescence staining pattern; 20–40% of the cells are strongly positive (+++, ID_{50} = 0.8 ng/ml) and 60% are weak or negative (−, ID_{50} = 250 ng/ml). Immunofluorescence of LNCaP cells with PR1(Fv)-PE38KDEL was weaker than observed with mAb PR1(+). ND, not done.

amined on several carcinoma cell lines. Fig. 5 and Table 1 show the cytotoxic effect of the recombinant immunotoxin toward mAb PR1-positive LNCaP cells. Because LNCaP cultures contained only 1–3% PR1-positive cells, we used a FACS to obtain a population composed of ≈40% strongly antigen-positive cells; the rest contained very little or no detectable antigen when analyzed by immunofluorescence with mAb PR1. By using these enriched LNCaP cells, the cytotoxicity curve is biphasic. About 40% of the cells are very sensitive to the recombinant immunotoxin with an ID_{50} of ≈0.8 ng/ml. The other 60% are more resistant (ID_{50}, 250 ng/ml). This was the expected result since only ≈40% of the

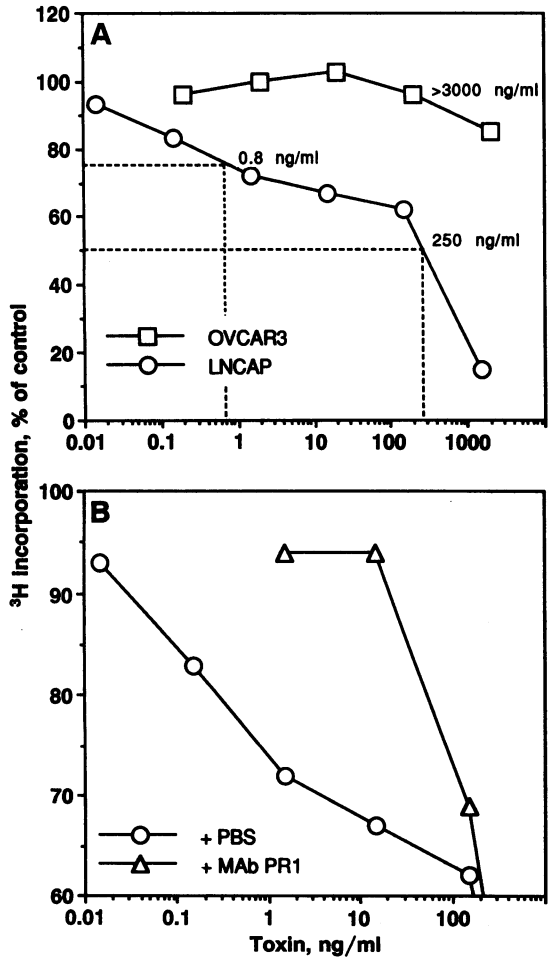

FIG. 5. Cytotoxicity of PR1(Fv)-PE38KDEL toward various carcinoma cells. (*A*) Toxicity of PR1(Fv)-PE38KDEL. (*B*) Inhibition of toxicity of PR1(Fv)-PE38KDEL by mAb PR1 (IgM) on PR1-positive LNCaP cells. Approximately 40% of the cells show strong expression in immunofluorescence and ID_{50} values of ≈0.8 ng/ml; 60% show weak expression and are more resistant to the immunotoxin.

LNCaP cells were reactive with mAb PR1. The cytotoxic effect of PR1(Fv)-PE38KDEL is specific because excess PR1 (15 μg/ml) blocked the cytotoxic effect but a control IgM (13F5) did not (Fig. 5). Furthermore, antigen-negative cells such as the ovarian cancer cell line OVCAR3 were not affected by the recombinant immunotoxin (Table 1).

Assay of PR1(Fv)-PE38KDEL by Magnetic Sorting. To analyze whether the recombinant PR1(Fv) immunotoxin can specifically eliminate antigen-positive cells from a mixed population of cells, we incubated LNCaP cultures, in which immunofluorescence with mAb PR1 showed that ≈10% of the cells contained the PR1 antigen, with immunotoxin for 40 hr. Then, the cultures were radiolabeled for 5 hr and remaining antigen-expressing cells were isolated with PR1-coated magnetic beads. Fig. 6 shows that PR1(Fv)-PE38KDEL is very cytotoxic toward PR1-expressing LNCaP cells. The ID_{50} was ≈3 ng/ml (the mean of four experiments). When antigen-negative OVCAR3 control cells were sorted in the same manner with OVCAR-3-specific 13F5(IgM)-coated beads, they were not affected. The cytotoxicity of PR1(Fv)-PE38KDEL toward LNCaP prostate carcinoma cells is specific because the cytotoxicity of the recombinant toxin was inhibited by addition of excess mAb PR1 (data not shown).

DISCUSSION

We have isolated mAb PR1 that reacts with an antigen on the surface of human prostate cancer cells and normal human prostatic cells and have cloned cDNAs encoding the V_H and V_L regions of mAb PR1. By using these cDNAs, a recombinant immunotoxin was constructed by connecting the V_H region to V_L by a $(Gly_4\text{-}Ser)_3$ linker and fusing this single-chain Fv to PE38KDEL (18), a truncated derivative of PE. The single-chain immunotoxin PR1(Fv)-PE38KDEL is specifically cytotoxic to prostate carcinoma cells that express the PR1 antigen on their surface.

In studies using human tissue specimens, mAb PR1 was found to bind to normal prostate epithelial cells and to prostate carcinoma cells. However, by using cell lines, the antigen was only found to be present in 1–3% of LNCaP cells and very few PC3 cells. Therefore, we used a FACS to obtain an enriched population of LNCaP cells for cytotoxicity and fluorescence experiments. Using this approach, we have obtained cultures of LNCaP cells that are up to 40% PR1-positive, but upon passage revert to 1–3%. Because 18 of 19 primary prostate cancers we have examined are strongly PR1-positive and 2 of 2 metastic lesions are also positive, it

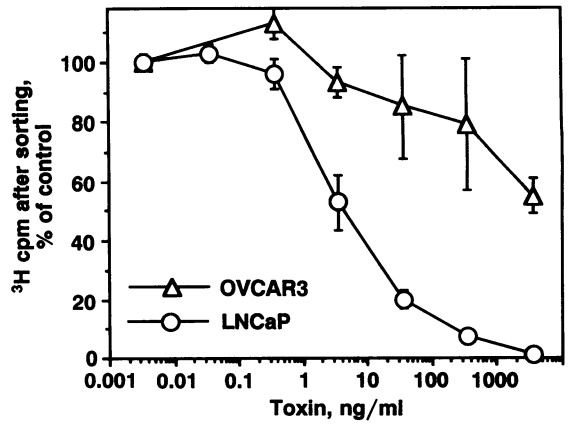

FIG. 6. Specificity of PR1(Fv)-PE38KDEL toward PR1-antigen-expressing LNCaP cells, determined by sorting with antibody-coated magnetic beads. Approximately 5 × 10^5 cells, grown in T25 flasks, were incubated with various concentrations of toxin for 40 hr, then labeled for 5 hr with [^{3}H]leucine, and sorted with either PR1-coated (LNCaP) or 13F5-coated (OVCAR3) magnetic beads.

is possible that the prostate carcinoma cell lines LNCaP and PC3, which contain a small percentage of PR1-positive cells, and cell line DU145, which has no detectable PR1 expression, may have lost expression during their establishment in culture.

Immunotoxins are usually made by chemically coupling toxins to the IgG form of an antibody. Because PR1 is an IgM, we were unable to make and purify an active immunotoxin using chemical-coupling methods. Furthermore, because of their large size, immunotoxins made with an IgM would be expected to penetrate tumors poorly (19, 20). We have tried to isolate an IgG switch variant of PR1 but have been unable to do so to date. Therefore, we set out to clone the variable regions of mAb PR1 and use these to construct the single-chain immunotoxin described here. These variable regions can also be used to make recombinant Fab fragments, which are useful for radioimaging, radiotherapy, or making drug conjugates.

We confirmed that the sequences we had cloned encode the variable regions of mAb PR1 by initially comparing the V_L-encoding DNA sequence to the amino-terminal protein sequence obtained from purified mAb PR1 κ chain. The heavy-chain protein sequence could not be obtained, because it was blocked against Edman degradation. We confirmed the authenticity of the cloned V_H sequence by directly sequencing mRNA from the PR1 hybridoma and found that the cloned V_H sequence is identical to the only IgM heavy-chain mRNA we found in the hybridoma. Homology analyses showed that PR1 V_L is almost identical to the light chain of the anti-idiotype mAb A25.9.7 (14). Surprisingly, the V_H sequence was very similar to another mAb, K1 (15), isolated in this laboratory. This was unexpected because heavy chains in contrast to light chains usually show a high degree of variability. However, no cross reactivity between the two anti-carcinoma antibodies was observed. mAb PR1 binds strongly to prostate tissues and carcinomas and not to OVCAR3 cells; K1 does not bind to prostate tissues but does bind strongly to OVCAR3 cells. Whether the V_H regions of mAbs K1 and PR1 are similar because both antibodies came up "early" in immunizations and are close to a germ-line sequence (K1 was originally isolated as an IgM like PR1, but subsequently isotyped switched) or recognize similar structures such as a similar antigen backbone remains to be clarified.

PR1(Fv)-PE38KDEL binds with the same specificity as mAb PR1 and its binding can be inhibited by mAb PR1, confirming that the bound antigen is the same. However, immunofluorescence signals with PR1(Fv)-PE38KDEL are weaker than with mAb PR1 (IgM). Due to our experience with other single-chain Fv molecules (1, 4, 10), we feel that the decreased signal is not caused by the peptide connecting the V_H and V_L domains or deletion of constant region κ or C_H1. The reduced binding probably reflects, at least in part, the difference between monovalent (Fv) and multivalent (IgM) binding. Lower immunofluorescence signals with Fab fragments obtained by proteolytic digestion of PR1 (data not shown) are in accordance with this conclusion. In addition, the presence of an N-glycosylation sequence (NYT) in complementarity-determining region 2 of the PR1 V_H (Fig. 2*A*) indicates that PR1 (IgM) might be glycosylated in this region. It has been shown that glycosylation of the complementarity-determining regions of antibodies can contribute to antigen binding (21). Because proteins from *E. coli* are not glycosylated, the recombinant immunotoxin may have reduced binding and activity.

PR1(Fv)-PE38KDEL is selectively toxic to LNCaP carcinoma cells expressing the PR1 antigen. The ID_{50} of PR1(Fv)-PE38KDEL on antigen-positive cells is between 0.8 and 3.0 ng/ml. Immunotoxins with this activity level have been found to cause complete regression of antigen-positive tumors in mice (10). Because the PR1 antigen is strongly expressed in prostate carcinomas and normal prostate, but only in few other normal tissues, we believe PR1(Fv)-PE38KDEL should be considered for use in humans with prostate cancer.

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