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Neutralization of Bleomycin Hydrolase by an Epitope-Specific Antibody

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SUMMARY

Bleomycin hydrolase (BH) is a cysteine proteinase that terminates the pharmacological action of bleomycin (BLM). Amino acid sequence data obtained from a tryptic digest fragment of purified rabbit lung BH were used to synthesize a 14-amino acid peptide (LAVLEQEPIVLPAK; BHP14), which was conjugated to horseshoe crab hemocyanin and used to produce rabbit antiserum that was immunoreactive to both BHP14 and rabbit BH. Anti-BHP14 binding to BHP14 could be competitively blocked by the presence of either BHP14 or BH. Anti-BHP14 recognized both purified rabbit liver BH and postmicrosomal fraction from rabbit liver on Western blot, as a single band of $M_r \approx 48{,}000$. Anti-BHP14 inhibited, in a concentration-dependent manner, BH activity in rabbit liver cytosolic fractions, as measured by deamido-BLM A_2 formation. Thus, we have generated an epitope-specific neutralizing antibody to rabbit BH, which can block the metabolism of BLM by homogenates from rabbit tissue. These results suggest that the LAVLEQEPIVLPA epitope of rabbit BH is involved in the metabolism of BLM or is topologically near the active site. Furthermore, a BLM-resistant squamous carcinoma (C-10E) exhibited slightly more immunoreactivity, by enzyme-linked immunosorbent assay, to anti-BHP14 than did the parental A-253 cells, and a partially revertant (C-10E ND) cell line had intermediate anti-BHP14 binding. BH activity in these cell lines was in the same rank order as antibody binding, but differences in immunoreactivity were less than differences in enzymatic activity. Our epitope-specific neutralizing antibody should be useful in the further characterization of BH.

The glycopeptide BLM is an essential component of the current combination chemotherapy used against a variety of solid tumors, including testicular and squamous cell carcinomas and malignant lymphomas (1). The pharmacological action of this DNA-cleaving agent may be terminated by the conversion of BLM to deamido-BLM by the cysteine proteinase BH, which is found primarily in the postmicrosomal supernatant fraction (2–4). It has been hypothesized that sensitivity of some tumors to BLM depends directly on the levels of BH in these tumors (5, 6). Furthermore, the clinical usefulness of BLM is limited by pulmonary toxicity, and vulnerability of lungs to BLM is thought to be inversely related to BH activity in this tissue (7, 8). Rabbit liver and lung BH have been purified and partially characterized previously (9, 10), but similar information on BH from other species is lacking. Molecular cloning and partial sequencing of BH have revealed membership in the cysteine proteinase family (11); furthermore, specific inhibitors of cysteine proteinases have been shown to augment the antitumor activity of BLM toward a variety of tumor cells (6, 12, 13). These results support a key role for this enzyme in the catabolism of BLM, as well as a possible role for BH in cellular resistance to BLM. We report here the development of an epitope-specific antibody directed against a synthetic polypeptide whose sequence was derived from a tryptic digest of purified rabbit lung BH. We have characterized this antibody and used it to determine whether BH or a BH-like protein participates in the metabolism of BLM.

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Experimental Procedures

Cell culture conditions. Human head and neck squamous carcinoma cells were maintained in culture as previously described (14). The C-10E cell line (derived from A-253) is 40-fold resistant to BLM, compared with the parental A-253 cells (13). Partially BLM-revertant

ABBREVIATIONS: BLM, bleomycin; deamido-BLM, deamido-bleomycin; BH, bleomycin hydrolase; HPLC, high performance liquid chromatography; BHP14, bleomycin hydrolase peptide; ELISA, enzyme-linked immunosorbant assay; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TTBS, Tris-Tween-buffered saline; PBS, phosphate-buffered saline; anti-BHP14, purified immunoglobulin against BHP14; E-64, *trans*-expoxysuccinyl-L-leucilamido-(4-guanidino)butane.

C-10E ND cells were derived from C-10E cells that were grown in the absence of drug for >2 months, and these cells are 13-fold resistant to BLM (15).

Peptide sequence analysis and conjugation reaction. Rabbit lung BH was purified to homogeneity (10) and subjected to cleavage by trypsin and HPLC separation as previously described (11). Based on the amino acid sequence of peak 58 (Fig. 1) determined with a gas-phase amino acid sequencer (model 470A; Applied Biosystems), we synthesized, at the Pittsburgh Cancer Institute Peptide Facility (Pittsburgh, PA), the peptide LAVLEQEPIVLPAK (BHP14), which contained a carboxyl-terminal lysine for ease of conjugation. The amino acid sequence for BHP14 was confirmed using an integrated microsequencing system (Porton Instruments). We then conjugated the peptide to horseshoe crab (*Limulus polyphemus*) hemocyanin, as follows. A solution containing 1.2 mg of BHP14 in 100 μl of water was mixed with another containing 50 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide in 100 μl of water. The mixture was allowed to react for 5 min, during which time the solution became slightly turbid. This was then mixed with a solution of 6.25 mg of horseshoe crab hemocyanin dissolved in 200 μl of water. This mixture was allowed to react for 25 min at 25°; the mixture was then placed in presoaked dialysis tubing and the volume was increased to 5 ml with water. The reaction mixture was dialyzed twice for 24 hr (4°) against 2 liters of distilled water; the reaction product was then lyophilized and stored in a dessicator at 4°.

Immunization of rabbits. Three New Zealand albino rabbits were inoculated with conjugated BHP14, after blood was drawn for preimmune sera on the day of the first injection (week 0). Each rabbit received 500 μg of conjugated BHP14 dissolved in 1 part PBS and 2 parts Freund's complete adjuvant for the initial injection. Rabbits were bled for sera every second week and boosted with 100 μg of conjugated peptide in PBS and Freund's complete adjuvant every fourth week. When ELISA indicated that serum immunoreactivity to BH had peaked, rabbits were sacrificed and blood was collected by cardiac puncture. Sera were frozen in 1.5-ml aliquots for subsequent processing.

ELISA. Immunoreactivity of sera from rabbits was tested using standard ELISA methodology. Briefly, 96-well MICROELISA plates (Dynatech) were precoated overnight with 100 μl of BHP14 or BH (100 ng/well). Plates were rinsed three times with TTBS (0.05% Tween-20, 0.15 M NaCl, in 10 mM Tris buffer, pH 7.4) and then blocked for 1 hr with 200 μl of Tris-buffered saline/10% nonfat dry milk. Plates were then rinsed three times with TTBS/1% nonfat dry milk. Sera (100 μl) from all bleedings, diluted 1/100 in TTBS/2% nonfat dry milk, were allowed to react with precoated antigen for 2 hr at 37°. The plates were rinsed three times with TTBS, 100 μl of goat anti-rabbit peroxidase-labeled IgG (Hyclone) diluted 1/3000 in Tween-PBS were added to each well, and the plates were incubated at room temperature for 1 hr. Plates were rinsed twice with TTBS and once with water. Into each well were pipetted 100 μl of *ortho*-phenylene diamine chromaphore (HyClone) dissolved in a 0.1 M citrate buffer/0.1% urea peroxide (HyClone) solution (pH 4.7). The plates were covered, incubated for 15 min to allow the color to develop, and read on a Titertek Multiskan plate reader at 492 nm.

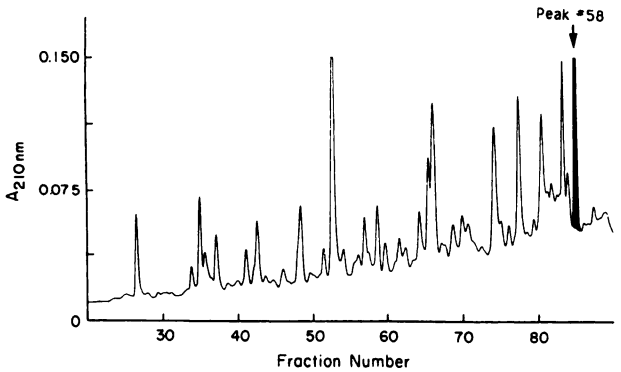

Fig. 1. Reverse phase HPLC profile of tryptic digest peptides of BH. Rabbit lung BH was purified to homogeneity as described previously (10). The purified enzyme (25 μg) was cleaved with trypsin, and the resulting peptide fragments were separated by reverse phase HPLC with an Aquapore column. Peak 58 was isolated and sequenced.

Purification of immunoglobulin specific for BH. Sera from each bleed were tested by ELISA (see above) for immunoreactivity against BHP14, and 6 ml of the highly immunoreactive sample from week 10 were dialyzed overnight at 4° against PBS (2 liters). The dialyzate was purified further on a Protein A-Sepharose (Pharmacia) affinity column, eluting with 6 ml of 0.1 M glycine (pH 3.0) solution. Fractions collected during glycine elution were immediately neutralized by addition of 3 M Tris buffer (pH 12). Fractions with immunoreactivity against BHP-14 (anti-BHP14) were lyophilized and resuspended to a final volume of 2 ml. Sera from the same rabbit before the first injection (preimmune sera) were similarly processed with a Protein A-Sepharose column, to yield preimmune immunoglobulin.

Affinity-purified anti-BHP14 was also prepared. Briefly, 1 g of epoxy-activated Sepharose 6B (Sigma) was swollen and washed with distilled water and then allowed to react with 36.8 mg of BHP14, dissolved in 10 ml of 0.1 M $NaHCO_3$, 0.5 M NaCl (pH 10.8) (coupling buffer), for 22 hr at 37°. The gel was washed several times with distilled water and then treated with 15 ml of 1 M ethanolamine (pH 7.8) for 2.5 hr, to block excess reactive epoxide groups. The slurry was then loaded onto a column and washed with coupling buffer, acetate buffer (pH 4.0), and PBS. Anti-BHP14 (1 ml; purified with Protein A-Sepharose as described above) was applied and eluted with 9 ml of triethylamine (pH 11.9), and the resulting fractions were immediately neutralized with NaH_2PO_4. After dialysis, the immunoreactive fractions were concentrated by centrifugation with Centriprep-10 (Amicon) concentrators, to yield affinity-purified anti-BHP14.

BH activity and inhibition with anti-BHP14. To measure BH activity, we homogenized and assayed rabbit liver as previously described (6, 10). Protein content was determined according to the method of Bradford (16). Metabolite peaks were visualized by either UV/visible or fluorescence spectrophotometry, and the peaks were integrated by a Waters base-line 810 program. For studies concerning metabolism by rabbit liver postmicrosomal fraction, the percentage of deamido-BLM A_2 formed in the presence of antibody was normalized to the percentage of deamido-BLM A_2 formed in the absence of antibody (100%). Control samples included BLM A_2 incubated in the absence of any source of BH and rabbit liver postmicrosomal fraction incubated in the absence of BLM A_2.

Western blotting. For Western blot analysis, SDS-PAGE was performed according to the method of Laemmli (17). Briefly, samples were heated at 95° for 3 min in sample buffer comprising 0.625 M Tris buffer (pH 6.8), 10% glycerol, 5% 2-mercaptoethanol, 2% SDS, and 0.2% bromphenol blue. Samples were loaded into wells on a 10% polyacrylamide gel. After electrophoretic separation (18 mA for 21 hr), proteins were transferred to Immobilon-P transfer membranes (Millipore) with a Bio-Rad Trans-Blot cell (100 mA for 22 hr). The membrane was stained with Ponceau S solution (0.5% Ponceau S/1% acetic acid) for 5 min, and molecular weight markers were recorded. The membrane was destained with water and exposed to blocking buffer (1% nonfat dry milk in PBS) for 1 hr. The membrane strips were then exposed to anti-BHP14 or preimmune immunoglobulin, diluted 1/100 in blocking buffer, for 2 hr. For competition experiments, anti-BHP14 was preincubated with 1 mg/ml BHP14 overnight and again with 1 mg/ml BHP14 in blocking buffer for 2 hr. Strips were then washed four times with PBS and exposed to horseradish peroxidase-labeled secondary antibody (goat anti-rabbit IgG; Hyclone), diluted 1/1000 in blocking buffer, for 1 hr. Strips were washed with PBS and subsequently developed for 1 min in a solution containing 0.5% diaminobenzidine, 0.03% H_2O_2 and 0.02% $CoCl_2$ in PBS.

Results

Design of BHP14 and generation of anti-BHP14. Rabbit lung BH was purified to homogeneity and digested with

trypsin as described previously (10). An HPLC profile of the tryptic digest is shown in Fig. 1. One of the peptide peaks (peak 58; Fig. 1) from the tryptic digest was sequenced and found to be outside of the open reading frame of the previously described partial BH rabbit liver cDNA (11). BHP14 was then synthesized, conjugated to hemocyanin, and injected into rabbits, as described in Experimental Procedures. In one of three rabbits, development of immunoreactivity to BHP14 or BH occurred within 10 weeks and remained elevated for an additional 6 weeks, until the time of sacrifice. It is evident that immunoreactivity to BHP14 closely parallels immunoreactivity to rabbit BH (Fig. 2). Lower immunoreactivity to rabbit BH, compared with synthetic peptide, probably reflects the lower molar quantity of BH plated, relative to BHP14 (100 ng/well for each). It was on the basis of this profile that serum from week 10 was selected for antibody purification.

Immunoaffinity purification of anti-BHP14 and competitive binding studies. Purification of sera was performed on a Protein A-Sepharose column, as described in Experimental Procedures, and purified immunoglobulin was then tested in a competitive ELISA, to determine whether binding of anti-BHP14 to precoated BHP14 could be blocked (Fig. 3). BHP14 (1 μg/100 μl) decreased binding by 76%, whereas partially purified BH (1 μg/100 μl) blocked binding of antibody to BHP14 by 87%. Increasing the blocking concentration of BHP14 to 10 μg/100 μl (95% blockade) or 100 μg/100 μl (100% blockade) demonstrates that binding of antibody to BHP14 could be competitively inhibited by the peptide in a concentration-dependent manner.

Western blotting. Western blot analysis revealed binding of anti-BHP14 to a single protein band ($M_r \approx 48{,}000$), corresponding to rabbit liver BH (Fig. 4A). Preincubation of anti-BHP14 with BHP14 (Fig. 4A, *lane 2*) completely abolished binding of anti-BHP14 to rabbit liver BH. In parallel experiments, preimmune immunoglobulin did not bind BH (Fig. 4A, *lane 3*). Western blot analysis of the 105,000 × *g* supernatant fraction from rabbit liver (Fig. 4B) also revealed binding of affinity-purified anti-BHP14 to a single protein band ($M_r \approx 48{,}000$). Preincubation of antibody with 1 mg/ml BHP14 blocked this binding (Fig. 4B, *lane 2*); preimmune immunoglobulin did not bind to this protein (Fig. 4B, *lane 3*). Similarly, a single band with this apparent molecular weight was seen with the 105,000 × *g* supernatant fraction from human A-253 cells (data not shown).

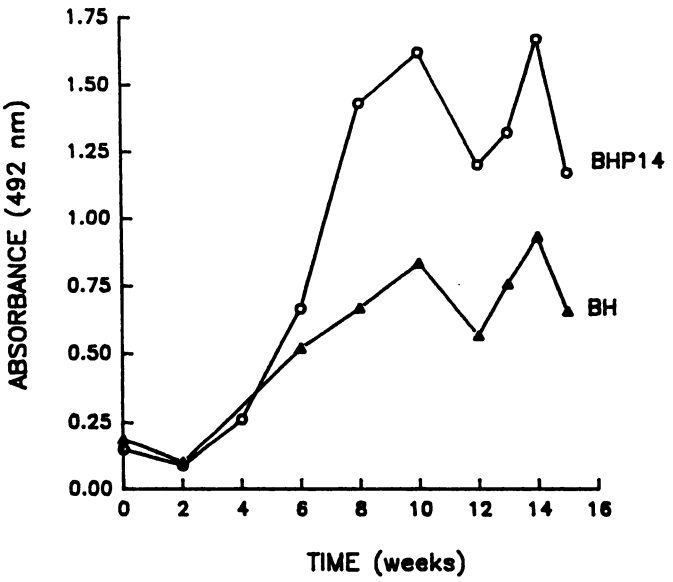

Fig. 2. Time course for development of antisera immunoreactive toward either BHP14 or BH. BHP14 was chemically linked to horseshoe crab hemocyanin as described in Experimental Procedures. Rabbits were inoculated with 500 μg of conjugated BHP14 initially and 100 μg of conjugated BHP14 every fourth week thereafter. Rabbits were bled before the first injection (week 0) and every second week thereafter. Sera were then tested for immunoreactivity toward either preplated BHP14 (○) or BH (▲), by ELISA.

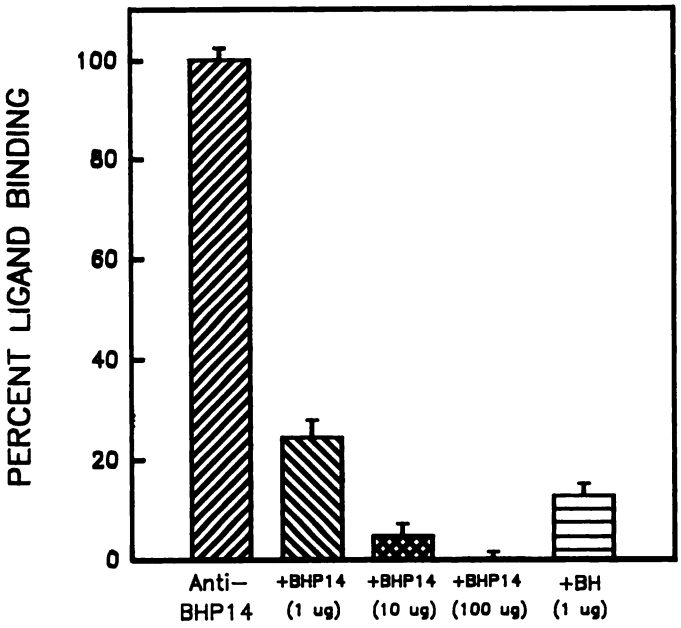

Fig. 3. Competitive binding assay between anti-BHP14 and either BHP14 or BH. A 96-well plate was precoated overnight with 100 ng of BHP14. The following day, binding of anti-BHP14 (diluted 1/100 in Tris-buffered saline with 0.1% gelatin) alone or anti-BHP14 incubated overnight in the presence of BHP14 (1, 10, or 100 μg/well) or BH (1 μg/well) was assessed by ELISA. Preimmune immunoglobulin was assessed concurrently, and the preimmune value ($A_{492} = 0.070$) was subtracted from all values. *Vertical bars*, standard error (eight experiments). All groups with BH or BHP14 were significantly different ($p < 0.05$) from the control group, as determined by Student's unpaired *t* test.

Effect of anti-BHP14 on BLM metabolism by rabbit liver. Anti-BHP14 incubated with rabbit liver as a source of BH neutralized BH activity, as determined by HPLC (Fig. 5). When rabbit liver postmicrosomal fraction as a source of BH was incubated in the absence of anti-BHP14 (Fig. 5A), a significant portion of BLM A_2 (Fig. 5A, *peak 1*) was converted to deamido-BLM A_2 (Fig. 5A, *peak 2*). Material eluting in the first 5 min coeluted with rabbit liver cytosolic fractions lacking BLM A_2 (data not shown). When the postmicrosomal fraction was incubated in the presence of 500 μg of anti-BHP14, markedly less BLM A_2 was converted to deamido-BLM A_2 (Fig. 5B). The formation of several minor peaks that eluted at 5–15 min and have not yet been chemically identified also was decreased. Indeed, BHP14 was found to be capable of inhibiting BH activity in a concentration-dependent manner (Fig. 6). Concentrations of anti-BHP14 as low as 50 μg inhibited BH activity. With the largest amount of anti-BHP14 added (500 μg), conversion of BLM A_2 to deamido-BLM A_2 was inhibited by 87%. Preimmune immunoglobulin purified by the same procedure as anti-BHP14 had no effect on BH activity.

Binding of anti-BHP14 and BH activity in human

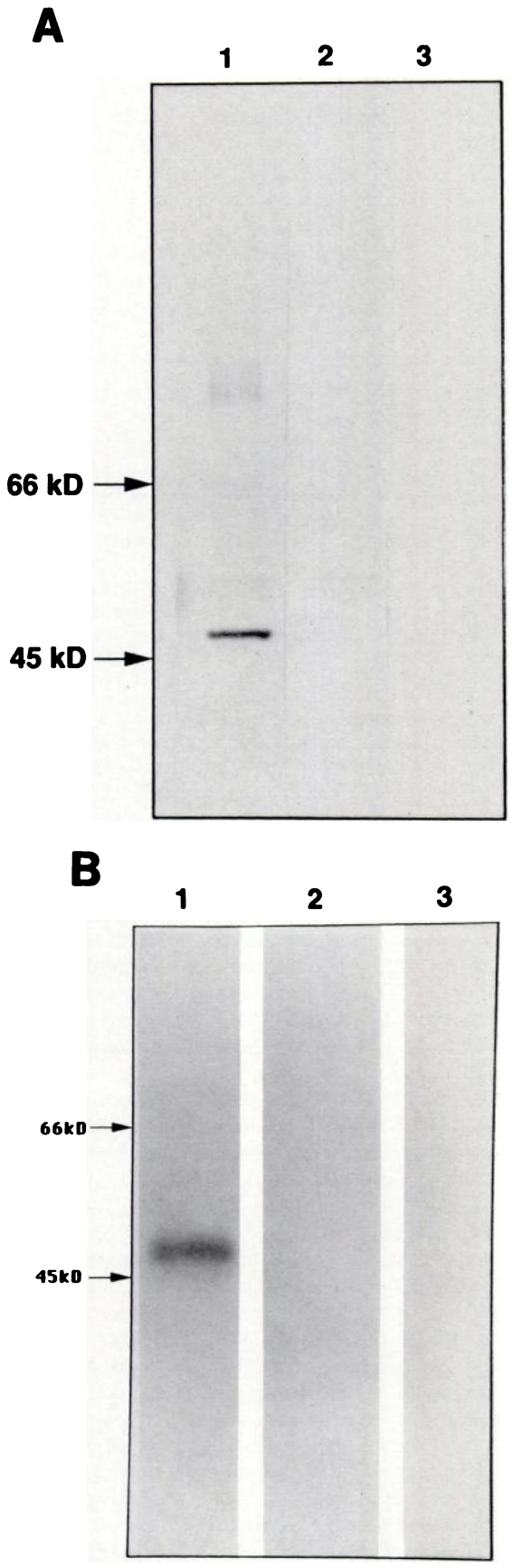

Fig. 4. A, Binding of anti-BHP14 to purified rabbit liver BH in Western blot. Rabbit liver BH, partially purified as described in Experimental Procedures, denatured and subjected to 10% SDS-PAGE at 100 mA for 22 hr. The following day, the proteins were transferred to an Immobilon polyvinylidene difluoride membrane at 100 mA for 22 hr. Protein markers were visualized with Ponceau S dye, and the membrane was processed by exposure to anti-BHP14 (*lane 1*), anti-BHP14 in the presence of 1 mg/ml BHP14 (*lane 2*), or preimmune immunoglobulin (*lane 3*). B, Binding of affinity-purified anti-BHP14 to rabbit liver postmicrosomal fraction. Rabbit liver was homogenized in Tris buffer (pH 7.4) and centrifuged at 105,000 × *g* for 1 hr. The supernatant was collected, and 100 μg/lane were loaded onto a 10% SDS-polyacrylamide gel, which was then handled as other Western blots (see above). The polyvinylidene difluoride membranes were then exposed to affinity-purified anti-BHP14 (*lane 1*), affinity-purified anti-BHP14 with 1 mg/ml BHP14 (*lane 2*), or affinity-purified preimmune immunoglobulin (*lane 3*).

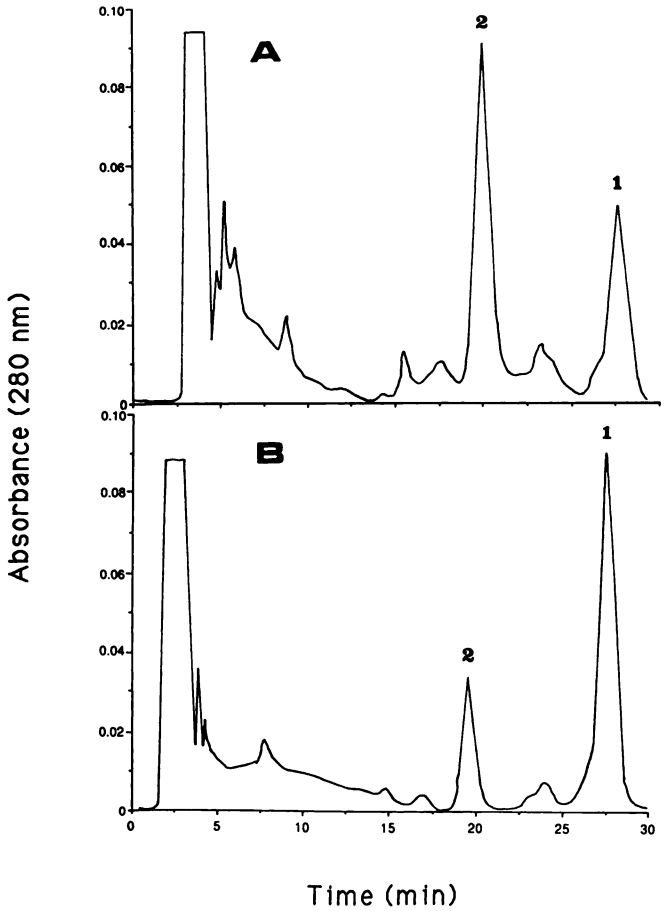

Fig. 5. Effect of anti-BHP14 on metabolism of BLM A_2 by rabbit liver. Rabbit liver postmicrosomal fraction (1 mg) was incubated with Cu^+-free BLM A_2 (21 μg) for 7 hr at 37°, in the absence (A) or presence (B) of 500 μg of BHP14. The reaction was terminated by the addition of 200 μl of methanol, and the samples were analyzed by C8 reverse phase HPLC, as described in Experimental Procedures. *Peak 1*, BLM A_2; *peak 2*, deamido-BLM A_2.

cancer cell lines. We next examined the ability of anti-BHP14 to react with the postmicrosomal (105,000 × *g*) supernatant fractions from a human head and neck squamous cell carcinoma (A-253) and two derived cell lines with different levels of drug sensitivity (Table 1). Binding of anti-BHP14 was least in BLM-sensitive A-253 cells, greatest in drug-resistant C-10E cells, and intermediate in partially revertant C-10E ND cells. BH activity was in the same rank order as immunoreactivity (Table 1); enzyme activity in drug-sensitive A-253 cells was 1.39 μg of BLM A_2 converted/mg of protein-hr, enzymatic activity was 3-fold higher in drug-resistant C-10E cells (4.46 μg of BLM A_2/mg of protein-hr), and activity was intermediate in

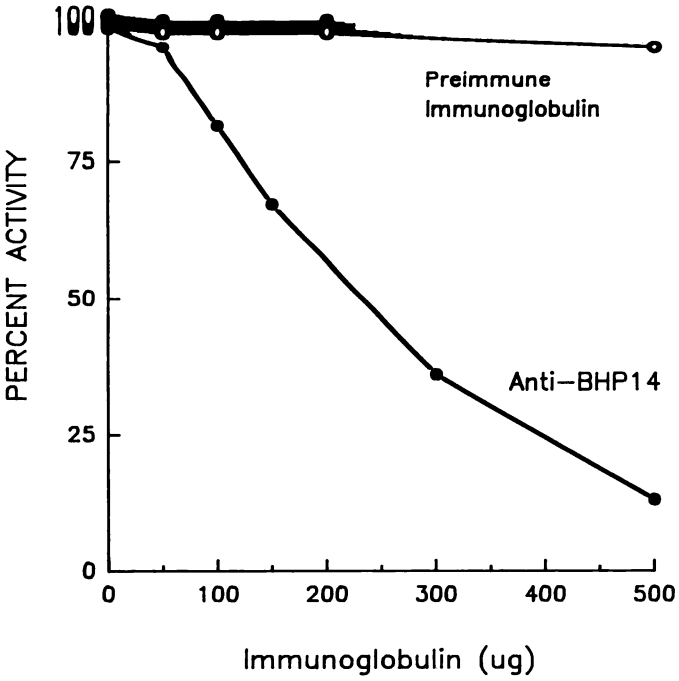

Fig. 6. Neutralization of rabbit liver BH with anti-BHP14. Rabbit liver postmicrosomal fraction (1 mg) as a source of BH was incubated for 7 hr at 37°, in the presence of increasing amounts of anti-BHP14. Each sample was analyzed separately by C8 reverse phase HPLC, and the percentage of BLM A_2 converted to deamido-BLM A_2 has been normalized to that obtained in the absence of immunoglobulin. In a separate reaction, preimmune immunoglobulin was utilized.

TABLE 1

Binding of anti-BHP14 and BH activity of human squamous cell carcinoma lines sensitive (A-253), partially revertant (C-10E ND), and resistant (C-10E) to BLM

Postmicrosomal fractions (8 μg of protein/well) were preplated into 96-well plates, and immunoreactivity was assessed by ELISA. Anti-BHP14 and preimmune immunoglobulin were diluted 1/200; the preimmune value (A_{492} = 0.037) was subtracted from all values. Affinity-purified anti-BHP14, prepared as described in Experimental Procedures, was diluted 1/20. Affinity-purified preimmune immunoglobulin was assessed concurrently, and the preimmune value (A_{492} = 0.051) was subtracted from all values. BH activity was assessed in postmicrosomal fractions from human cancer cells by incubating BLM A_2 (20 μg) with postmicrosomal fractions (250 μg of protein/sample) for 9 hr at 37°. Values are mean ± standard error for 8–16 determinations for anti-BHP14 binding and four determinations for BH activity.

Cell type	Immunoreactivity of anti-BHP14	Immunoreactivity of affinity-purified anti-BHP14	BH activity
	A_{492}	A_{492}	μg of BLM A_2 converted/mg-hr
A-253	0.080 ± 0.003	0.393 ± 0.016	1.39 ± 0.29
C-10E ND	0.090 ± 0.004[a]	0.448 ± 0.011[a]	3.23 ± 0.06[a]
C-10E	0.112 ± 0.002[a]	0.479 ± 0.011[a]	4.46 ± 0.63[a]

[a] $p < 0.05$ versus A-253.

the partially revertant C-10E ND cell line (3.23 μg of BLM A_2/mg-hr). All human postmicrosomal fractions converted BLM A_2 to a complex mixture of metabolites and, therefore, BH activity was based upon disappearance of BLM A_2.

Discussion

We report here the development of an epitope-specific antibody that was raised against a sequence of 14 amino acids from BH; this sequence is encoded by a region outside of the currently known partial length BH cDNA (11). Results from several studies suggest that this antibody recognizes rabbit BH. Firstly, the immunoreactivity of rabbit anti-BHP14 antisera toward BHP14 and rabbit BH paralleled each other throughout the course of immunization (Fig. 2). Secondly, competition experiments demonstrated that either BHP14 or BH was capable of blocking the binding of purified immunoglobulin to BHP14 (Fig. 3). With respect to BHP14, inhibition of binding was concentration dependent and could be completely blocked with an excess (100 μg) of BHP14. It is intriguing that binding could be blocked more effectively with native partially purified BH than with the synthetic peptide (BHP14) from which the antibody was raised. It is possible that BHP14 linked to hemocyanin forms a spatial epitope more like that found in native BH than does BHP14 alone. Finally, Western blot analysis revealed binding of Protein A-purified anti-BHP14 to a single band from partially purified rabbit liver BH (Fig. 4A) and binding of affinity-purified anti-BHP14 to a single band from rabbit liver postmicrosomal fraction (Fig. 4B). The bands appear at $M_r \approx 48{,}000$, the reported molecular weight for BH derived from rabbit liver and lung (9, 10). Together, these observations provide persuasive evidence that the antibody we have raised recognizes rabbit BH.

We have recently shown that BLM metabolism plays an important role in the resistance of some human tumors *in vivo* (6). In this report, we have quantified BH activity in these three squamous carcinoma cell lines for the first time (Table 1), and we show that acquired resistance to BLM is characterized by elevated BH activity. Using anti-BHP14 and homogenates of A-253 cells, we find a single band on Western blot, suggesting a conserved epitope in human tissue. Interestingly, whereas binding of anti-BHP14 in an ELISA is in the same rank order as enzymatic activity, the relative increase in immunoreactivity is less than the 3-fold increase in BH activity in the resistant C-10E cell line. Our results, therefore, support the hypothesis that the increased BH activity seen in BLM-resistant C-10E cells is due to altered enzyme or endogenous modulators of activity. It is known that, in most human tissues, cysteine proteinases are regulated by endogenous inhibiting proteins, such as cystatins (18). The anti-BHP14 should assist us in examining this question further.

Enzymatic activity studies suggest the 14-amino acid sequence (BHP14) to be an important functional region for BH. The addition of anti-BHP14 to rabbit liver supernatant fraction resulted in a concentration-dependent inhibition of BH activity; the antibody is, therefore, neutralizing (Fig. 6). At the highest concentration of anti-BHP14 added, only 13% residual activity was detected. The full length BH cDNA has not yet been isolated and, therefore, the complete amino acid sequence for rabbit BH is not yet known. Although the amino acid sequence used to generate antibody is not encoded by the previously reported partial length cDNA for rabbit BH, the sequence is outside of the predicted active catalytic site for cysteine proteinases (11). Thus, it is somewhat surprising that anti-BHP14 neutralizes BH activity. This suggests that the BHP14 epitope might be close to the active site of BH or that anti-BHP14 is capable of allosterically altering the active site when bound to BH or affecting the binding of some unknown cofactor. We have synthesized another, more hydrophilic, peptide (KSSGRCWIF), which encodes the putative active site, in an attempt to make neutralizing antisera, but we have been

unsuccessful in generating immunoreactive sera with this peptide.

Although the metabolism of BLM to deamido-BLM by BH has been extensively documented (7, 8, 12, 19–21), only recently have reports concerning the possible existence of other metabolites appeared. Using a recently developed high resolution HPLC system, our laboratories have noted the presence of multiple peaks in the analysis of BLM A_2 metabolism by Burkitt's lymphoma; moreover, these peaks were abolished by pretreatment with E-64, a cysteine proteinase inhibitor known to irreversibly block BH activity (6). Similarly, rabbit liver postmicrosomal fraction generated a number of metabolites distinct from deamido-BLM, and the formation of these metabolites was diminished in the presence of anti-BHP14 (Fig. 5). This raises two interesting possibilities; either these metabolites are formed from deamido-BLM A_2 or there are other enzymes responsible for BLM metabolism. In either case, these metabolites appear, like deamido-BLM, to be incapable of cleaving DNA (6). Anti-BHP14 also blocked the *in vitro* metabolism of BLM A_2 in human tissue, whereas preimmune immunoglobulin had no effect (data not shown). Although the enzymes responsible for metabolic inactivation of BLM A_2 in human tissue are not yet known precisely, our results indicate that human cells contain an amino acid sequence similar, if not identical, to that of peak 58 found in rabbit BH (Fig. 1). Our previous studies (6) also support the hypothesis that inactivation of BLM A_2 in human tissues is mediated by a BH-like enzyme containing a cysteine proteinase active site, because a specific cysteine proteinase inhibitor, E-64, completely blocked BLM A_2 metabolism in human tissue. This is particularly interesting because we have demonstrated that blockade of BLM metabolism with E-64 renders human tumor cells more vulnerable to BLM cytotoxicity (6, 13).

Previously, a monoclonal antibody to isolated rabbit liver BH had been used to purify the enzyme (9). Although it was neutralizing, neither the epitope of the previously described antibody nor its reactivity with human BH is known. Moreover, our antibody is useful for Western blotting and recognizes human tumor BH in three related cell lines, in the same rank order as their relative drug resistance (A-253 < C-10E ND < C-10E); however, antibody recognition suggests much smaller differences in immunoreactive protein than in enzyme activity. We, therefore, suggest that regulation of BH activity, either by post-translational modification of BH molecules or by endogenous inhibitors such as cystatins (18), contributes to the greater enzyme activity seen in the resistant cell lines. We anticipate that this antibody will be useful in efforts to purify BH, to localize BH within cells, and to understand BLM metabolism in normal and malignant tissues.

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