

# A Novel Ligand in Lymphocyte-mediated Cytotoxicity: Expression of the $\beta$ Subunit of $H^+$ Transporting ATP Synthase on the Surface of Tumor Cell Lines

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## Summary

Extracellular adenosine triphosphate (eATP) has been suggested to play a role in lymphocyte-induced tumor destruction. We now provide evidence that a protein responsible for ATP synthesis in mitochondria may also play a physiologic role in major histocompatibility complex-independent, lymphocyte-mediated cytotoxicity. A 51.5-kD protein (p51.5) bearing structural and immunologic characteristics of the  $\beta$  subunit of  $H^+$  transporting ATP synthase (E.C. 3.6.1.34,  $\beta$ - $H^+$  ATPase, published molecular mass of 51.6 kD) was detected on the plasma membrane of three different human tumor cell lines studied.  $NH_2$ -terminal amino acid sequence analysis of purified p51.5 from K562 tumor cells revealed 100% homology of 16 residues identified in the first 21 positions to the known sequence of human mitochondrial  $\beta$ - $H^+$  ATPase. Antibody directed against a 21-mer peptide in the ATP binding region of  $\beta$ - $H^+$  ATPase (anti- $\beta$ ) reacted with only one band on Western blots of whole tumor extracts and tumor membrane extracts suggesting that the antiserum reacts with a single species of protein. Anti- $\beta$  reacted with the cell membranes of tumor cells as determined by fluorescence-activated flow cytometry and immunoprecipitated a 51.5-kD protein from surface-labeled neoplastic cells (but not human erythrocytes and lymphocytes). Purified p51.5 bound to human lymphocytes and inhibited natural killer (NK) cell-mediated cytotoxicity. Furthermore, anti- $\beta$  treatment of the K562 and A549 tumor cell lines inhibited NK (by >95%) and interleukin 2-activated killer (LAK) cell (by 75%) cytotoxicity, respectively. Soluble p51.5 upon binding to lymphocytes retained its reactivity to anti- $\beta$  suggesting that the ATP binding domain and the lymphocyte-receptor binding domain reside in distinct regions of the ligand. These results suggest that  $\beta$ - $H^+$  ATPase or a nearly identical molecule is an important ligand in the effector phase (rather than the recognition phase) of a cytolytic pathway used by naive NK and LAK cells.

Lymphocytes that directly destroy target cells upon contact (cytolytic lymphocytes) play an important protective role against cancer and intracellular parasites such as viruses. The processes by which cytolytic lymphocytes destroy such cells is not completely understood. Current concepts view cytolysis as a multistep physiologic process minimally involving initial weak attachment of the lymphocyte to the target cell, specific recognition, transmembrane signaling that strengthens attachment, further bidirectional signaling events that ultimately program the target cell for destruction, lysis of the target cell, and recycling of the effector cell to another target

(for reviews see references 1–5). These processes are likely mediated by receptor–ligand interactions that may vary somewhat depending on the particular combination of effector and target cell and/or the cytolytic pathway used (5, 6).

Evidence for at least three separate lytic pathways has been demonstrated. One pathway involves exocytosis of lytic granules into the cleft formed by the lymphocyte and the target cell membranes (4, 7, 8). Another pathway is effector cell-induced target cell autolysis, i.e., apoptosis (9, 10). A third cytolytic pathway involves influx of ions and water into target cells leading to osmotic shock and cellular dis-

ruption (7, 11–13). Extracellular ATP (eATP)<sup>1</sup> has recently been suggested to play an important role in this latter pathway (14). Incubation of tumor cells with ATP induced Ca<sup>2+</sup> mobilization, plasma membrane depolarization, activation of phospholipase C, osmotic swelling, and cell lysis (15–18), characteristics commonly observed in tumor cells during lymphocyte-mediated cytotoxicity (11–13). It is significant that elevated levels of eATP are detected in the early phase of lymphocyte-mediated tumor cell death (19). The mechanisms by which eATP is generated and used during the cytolytic process have not been elucidated.

During investigation of cytolytic lymphocyte–target cell interaction, we identified a number of lymphocyte-binding tumor plasma membrane proteins (20). Based on structural and immunologic properties, we now report that one of these tumor membrane proteins has homology to the  $\beta$  (catalytic) subunit of H<sup>+</sup> transporting ATP synthase (E.C.3.6.1.34,  $\beta$ -H<sup>+</sup> ATPase) a molecule thought to be exclusively located in mitochondria (21). Functional assays suggest that  $\beta$ -H<sup>+</sup> ATPase is involved in a cytolytic pathway that functions in naive NK cells and IL-2-activated killer (LAK) cells.

## Materials and Methods

**Chemicals and Antibodies.** *N*-hydroxy succinamide ester of biotin (biotin-NHS) was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). Streptavidin-alkaline phosphatase and all cell culture media and reagents were from GIBCO BRL (Gaithersburg, MD). Chemicals used for electrophoresis were purchased from Bio-Rad Laboratories (Melville, NY). All other chemicals used were obtained from Sigma Chemical Co. (St. Louis, MO). Mouse anti-rabbit IgG was purchased from Pierce (Rockford, IL). Goat anti-rabbit IgG conjugated to FITC was obtained from Tago, Inc. (Burlingame, CA). Antibody against  $\beta$ -H<sup>+</sup> ATPase (anti- $\beta$ ) was kindly provided by Drs. P. J. Thomas and P. L. Pederson (Johns Hopkins University, Baltimore, MD). Anti- $\beta$  was raised in rabbits against a 21-mer peptide corresponding to the amino acid sequence Y311 to A331 of  $\beta$ -H<sup>+</sup> ATPase and had a titer of 1:50,000 (22).

**Cell Isolation and Culture.** Cell lines used in this study were obtained from the American Type Culture Collection (Rockville, MD) and were maintained in media according to their instructions. Human erythrocytes and human PBL (HPBL) were isolated as described previously (23, 24). Briefly, blood obtained from healthy volunteers was diluted 1:1 with HBSS, layered on Ficoll-Hypaque (Pharmacia, Piscataway, NJ), and centrifuged at 200 *g* for 20 min. The pelleted erythrocytes were washed three times with HBSS and used fresh. HPBL were depleted of B cells and monocytes by passage of the mononuclear leukocyte fraction (Ficoll-Hypaque interface layer) through nylon wool columns. LAK activity was generated by incubation of freshly isolated HPBL at 10<sup>6</sup> cells/ml with 30 U rIL-2/ml (R&D Systems, Inc., Minneapolis, MN) for 6 d at 37°C in 5% CO<sub>2</sub> (23, 24).

**Purification of Tumor Membrane Proteins.** Approximately 10<sup>9</sup> cells of erythroleukemia cell line (K562) were washed three times with 10 mM phosphate, pH 7.4, containing 145 mM NaCl (PBS)

and used to prepare plasma membranes as described previously (25). Plasma membrane proteins were solubilized with 1% Triton X-100 overnight at 4°C in a buffer containing 10 mM sodium borate, 10 mM benzamidine, 1 mM EDTA, 1 mM iodoacetamide, and 1 mM PMSF, pH 8.0 (buffer A). Protein concentrations were determined by the method of Bradford (26). Solubilized proteins were extensively dialyzed against buffer A and subjected to preparative SDS-PAGE (27). A vertical portion of the gel was sliced and stained with Coomassie blue to locate the desired protein. Protein bands of interest were cut from the gel, eluted, and further purified by SDS-PAGE. A portion of the purified protein was extensively dialyzed against 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0, and used either for cytotoxicity inhibition studies or labeled with biotin as described previously (25) for study of its binding properties to HPBL. A portion of the purified protein was also isolated on nitrocellulose paper by SDS-PAGE and transblotting (27, 28) and utilized for NH<sub>2</sub>-terminal amino acid sequence analysis (Yale University Microsequencing Laboratory, New Haven, CT).

**Immunoprecipitation.** Plasma membrane proteins of viable tumor cells were first labeled with biotin according to standard techniques (29, 30). Briefly, cells (>99% viable by trypan blue dye exclusion) were washed three times with a solution containing 10 mM Hepes, 145 mM NaCl, 4 mM KCl, 11 mM glucose, pH 8.0, (buffer B), and then incubated in the same buffer with biotin-NHS (1 mM biotin-NHS/10<sup>6</sup> cells) for 1 h at 4°C. Subsequently, cells were washed three times with 20 vol of buffer B to remove unreacted biotin, and plasma membrane proteins were prepared as described above.

Anti- $\beta$  was incubated with mouse anti-rabbit IgG coupled to CNBr-Sepharose 4B at 4°C for 90 min in PBS containing 1% BSA. The reacted beads were washed three times with buffer and incubated at 4°C for 90 min with biotinylated tumor plasma membrane proteins. The beads were then washed three times with PBS containing 1% BSA and two times with PBS alone, suspended in Laemmli SDS-PAGE sample buffer, and boiled for 10 min. The supernatant was subjected to SDS-PAGE (27) and proteins from gels were transferred to Immobilon-P membrane (Millipore Corp., Bedford, MA) by electroblotting (28). The immunoprecipitated biotinylated tumor membrane proteins were identified on blots by reaction with a streptavidin-biotin detection system (25).

**Binding of Tumor Plasma Membrane Proteins to Viable Lymphocytes.** Plasma membrane proteins of tumor cells or purified proteins were prepared as described above. Proteins were labeled with biotin and reacted with freshly isolated viable HPBL at a ratio of 2:1 (on the basis of cell numbers) in RPMI-1640 supplemented with 15% FCS at 4°C for 2 h. The coated HPBL were washed twice with media and three times with buffer B. Finally, cells were solubilized in Laemmli sample buffer and the solubilized proteins were subjected to SDS-PAGE and Western blotting. Biotinylated tumor membrane proteins that bound to lymphocytes were identified with a streptavidin-biotin detection system (25).

**Fluorescence-activated Flow Cytometry.** Approximately 10<sup>6</sup> cells were washed three times with PBS containing 0.1% BSA (PBS-BSA) and incubated with appropriately diluted anti- $\beta$  at 4°C for 30 min. Cells were then washed three times with PBS-BSA and reacted with FITC-F(ab')<sub>2</sub> goat anti-rabbit IgG for 30 min at 4°C. Finally, cells were washed three times with PBS-BSA, resuspended to a concentration of 10<sup>6</sup> cell/ml (viability > 95%), and analyzed on a cytofluorograph, model IIs (Ortho Pharmaceutical, Raritan, NJ).

**Lymphocyte-mediated Cytotoxicity.** Cytotoxicity of tumor cells by HPBL was measured as described previously (23, 24). In brief, 100  $\mu$ l of <sup>51</sup>Cr-labeled target cells (2 × 10<sup>6</sup> cells/ml) was mixed with

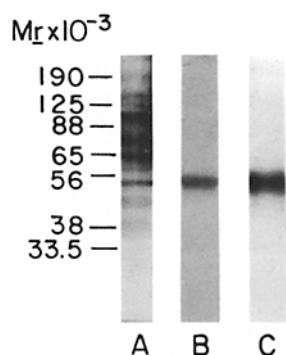
<sup>1</sup> Abbreviations used in this paper: biotin-NHS, *N*-hydroxy succinamide ester of biotin;  $\beta$ -H<sup>+</sup> ATPase,  $\beta$  subunit of H<sup>+</sup> transporting ATP synthase; eATP, extracellular ATP; HPBL, human peripheral blood lymphocytes; p51.5, a 51.5-kD plasma membrane protein of a human erythroleukemia cell line (K562).

100  $\mu$ l of HPBL ( $2 \times 10^6$  cells/ml) to yield serial twofold dilutions of E/T cell ratios of 100:1 to 6:1. Cells were incubated at 37°C for 3 h in 5% CO<sub>2</sub>. Incubation of target cells without the effector cells and in media alone served as control for spontaneous release of <sup>51</sup>Cr, and wells without effector cells but with 1% SDS provided the maximum amount of radioactivity present. Cytotoxicity was calculated as percent cytotoxicity =  $100 \times [(cpm_{exp} - cpm_{spont}) / (cpm_{max} - cpm_{spont})]$ .

## Results

**Binding of Tumor Plasma Membrane Protein p51.5 to HPBL.** To identify tumor membrane proteins that bind to lymphocytes, we adopted a technique that was successfully utilized to characterize receptor-ligand interactions between *Staphylococcus aureus* and human endothelial cell plasma membrane proteins (25). Initial studies were performed with the NK-sensitive K562 tumor cell line. Solubilized membrane proteins from surface biotin-labeled K562 cells were reacted with freshly isolated HPBL. Western blots of these solubilized HPBL preparations revealed a number of lymphocyte-binding tumor membrane proteins (Fig. 1, lane A). Protein of one of the major bands (molecular mass 51–55 kD) was purified by preparative gel electrophoresis from K562 membrane extracts for further study. The purified K562 protein appeared as a single band of 51.5-kD molecular mass (p51.5) upon SDS-PAGE (Fig. 1, lane B). The binding property of purified p51.5 was examined by an identical protocol used to identify lymphocyte-binding tumor proteins from crude membrane preparations. Representative experiments demonstrated that purified p51.5 retained its lymphocyte-binding property (Fig. 1, lane C).

**Effect of Purified p51.5 on NK Cell-mediated Cytotoxicity.** Since p51.5 from the prototypical NK target cell bound to the plasma membrane of HPBL, we hypothesized that it might function as a ligand in the cytolytic process. To test this proposal, a NK cytotoxicity assay system was utilized where freshly isolated HPBL were cultured with purified p51.5 before and during incubation with <sup>51</sup>Cr-labeled K562 target cells. We



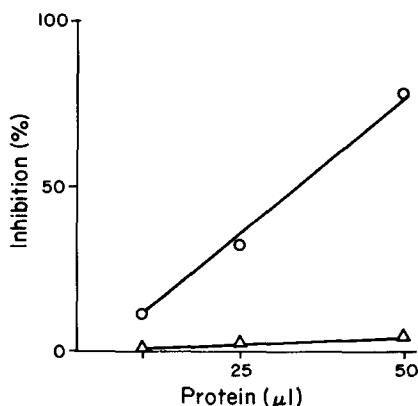
**Figure 1.** Binding of K562 membrane proteins to HPBL. (Lane A) A representative blot of surface-labeled K562 membrane proteins that bind to HPBL. K562 membrane proteins from surface biotinylated cells were reacted with freshly isolated viable HPBL. The treated HPBL were washed with buffer, solubilized in Laemmli sample buffer, and proteins resolved by SDS-PAGE. Biotinylated tumor plasma membrane proteins that bound to HPBL were detected on Western blots by a standard streptavidin-alkaline phosphatase detection system. (Lane B) A Western

blot of Coomassie blue-stained purified p51.5 K562 membrane protein. (Lane C) A Western blot of a HPBL extract derived from viable HPBL that were reacted with purified biotin-labeled p51.5. The binding reaction was performed with purified p51.5 by the same method as used for lane A experiments.

reasoned that under these experimental conditions the ligand binding site of the putative p51.5 NK receptor would be blocked by soluble p51.5, thereby preventing engagement of the “physiologic” ligand on K562 cells. Indeed, significant inhibition in NK-mediated cytotoxic activity was observed when p51.5-treated HPBL were used as effector cells compared with untreated lymphocytes (Fig. 2). The extent of inhibition was dependent on the concentration of purified p51.5 reacted with the HPBL. An 80-kD K562 membrane protein (which did not bind to HPBL) was purified and studied for its ability to block NK activity in an identical manner to p51.5. As shown in Fig. 2 the 80-kD membrane protein was unable to block NK-mediated cytotoxicity.

**Structural Characterization of p51.5.** To investigate the identity of p51.5, NH<sub>2</sub>-terminal amino acid sequence analysis was performed on SDS-PAGE-purified protein as described above. Of the first 21 cycles, a single amino acid was identified at each of 16 positions with a high degree of confidence (Table 1). This sequence was compared with known sequences in GenBank (see Table 1). The search revealed 100% identity at each of these positions with residues at the NH<sub>2</sub>-terminus of the  $\beta$  subunit of  $\beta$ -H<sup>+</sup> ATPase, a molecule that is part of a multimeric mitochondrial enzyme complex (21). A comparison of quantitative data from the sequenced amino acids and from total protein amino acid analysis suggested that the sequence was derived from the major species in the preparation which account for >95% of the total protein.

The origin of  $\beta$ -H<sup>+</sup> ATPase in the crude membrane preparations used in the above studies could be ascribed solely to contamination of these extracts with mitochondria. This proposal, however, does not explain the observations that surface-labeled p51.5 bound to HPBL (Fig. 1) and inhibited NK cell-mediated cytotoxicity in contrast to another tumor membrane protein (Fig. 2). These latter data strongly suggested



**Figure 2.** Effect of purified K562 membrane proteins on NK cell-mediated cytotoxicity. HPBL were first incubated with the indicated amount of purified proteins (40 ng/ $\mu$ l based on amino acid analysis) at 37°C for 30 min and then added to <sup>51</sup>Cr-labeled K562 cells at an E/T ratio of 100:1. Percent cytotoxicity was determined after 3-h incubation by the release of <sup>51</sup>Cr and the data expressed as percent inhibition relative to a buffer control. Percent cytotoxicity against K562 cells with buffer alone was 55%. (O-O) p51.5 and (Δ-Δ) p80 K562 membrane proteins. Representative data of three experiments is shown.

**Table 1.** *NH<sub>2</sub>-terminal Amino Acid Sequence of p51.5 K562 Membrane Protein: Comparison with the Known Sequence of Human  $\beta$ -H<sup>+</sup>ATPase*

	1	10	20
51.5-kD-K562 Membrane protein (p51.5)	XXQTXPKAGXATGXIVAVI		
$\beta$ -H <sup>+</sup> ATPase (EC 3.6.1.34)	AAQTSPSPKAGAATGRIVAVI		

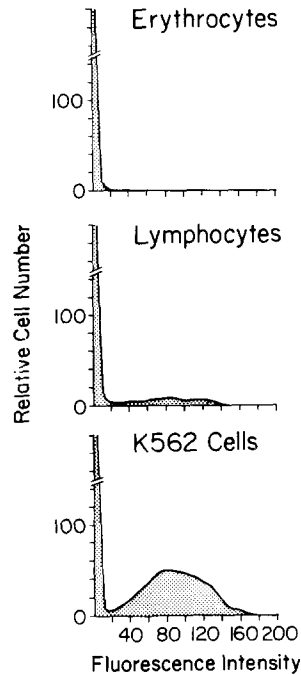
Amino acid sequence analysis was performed on p51.5 purified by preparative SDS-PAGE as described in Materials and Methods. Amino acid at position X could not be identified with high certainty. The sequence of the p51.5 peptide was compared to  $\beta$ -H<sup>+</sup>ATPase by GenBank, Sequence Analysis Software Package, Genetics Computer Group (University Research Park, Madison WI).

that a  $\beta$ -H<sup>+</sup>ATPase-like molecule is expressed on the surface of tumor cells. Accordingly, immunologic studies were conducted to establish whether a  $\beta$ -H<sup>+</sup>ATPase-like molecule (p51.5) is expressed on the exterior of tumor cell plasma membranes. Additionally, immunologic characterization of the molecule that bound to HPBL was performed.

**Immunologic Characterization of K562 Plasma Membrane p51.5.** Affinity-purified antibody directed against a peptide in the ATP-binding region of  $\beta$ -H<sup>+</sup>ATPase (anti- $\beta$ , see Materials and Methods) was used to study the expression of  $\beta$ -H<sup>+</sup>ATPase on the plasma membrane of representative tumor cell lines and normal cells by fluorescence-activated flow cytometry and immunoprecipitation. Viable cells were reacted with anti- $\beta$ , and then reacted with FITC-F(ab')<sub>2</sub> goat anti-rabbit IgG. Under these conditions, anti- $\beta$  bound to ~90% of K562 cells but did not bind substantially to HPBL or human erythrocytes (Fig. 3). Flow cytometry analysis of a human lung adenocarcinoma cell line (A549) and a Burkitts lymphoma cell line (Raji) also demonstrated >90% reactivity with anti- $\beta$  (data not shown). These results suggest the presence of a molecule on the surface of a variety of tumor cell lines (but not normal human lymphocytes and erythrocytes) that has immunologic identity or cross-reactivity to  $\beta$ -H<sup>+</sup>ATPase.

Western blots of K562 extracts were probed to determine the number of proteins that might cross-react with anti- $\beta$ . It is significant that anti- $\beta$  reacted with only a single protein band of 51.5 kD on the Western blots of whole K562 cell extracts as well as extracts of K562 plasma membrane (Fig. 4). Identical results were obtained with A549, Raji, and HPBL. Serum from nonimmune rabbits or rabbits immunized against an irrelevant protein lacked reactivity against p51.5 (data not shown). These findings strongly suggest that anti- $\beta$  does not react with cellular proteins other than  $\beta$ -H<sup>+</sup>ATPase.

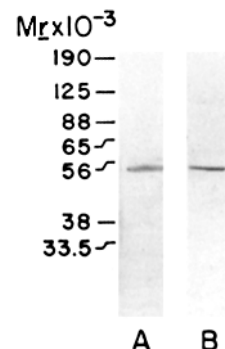
To directly demonstrate that the Y311 to A331 epitope of  $\beta$ -H<sup>+</sup>ATPase is expressed by a 51.5-kD tumor protein of plasma membrane origin, solubilized membrane protein from



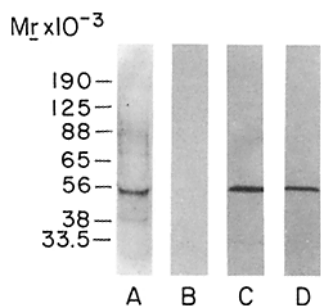
**Figure 3.** Flow cytometry analysis of anti- $\beta$ -treated cells. Lymphocytes and K562 cells treated with secondary antibody alone (i.e., goat anti-rabbit F(ab')<sub>2</sub> IgG-FITC) were <5% positive. Data are representative of three experiments.

surface biotin-labeled K562 cells or control HPBL were reacted with anti- $\beta$ . (Antibody directed against the above epitope, which constitutes a portion of the ATP-binding domain, is important for the catalytic function of  $\beta$ -H<sup>+</sup>ATPase; 22). A single biotinylated protein of 51.5 kD was detected in the immunoprecipitate of the K562 membrane preparation, whereas the immunoprecipitate from HPBL did not reveal biotinylated protein from the cell surface (Fig. 5, lanes A and B, respectively). Immunoprecipitation studies with anti- $\beta$  also demonstrated plasma membrane-reactive protein (51–55 kD) on A549 and Raji cell lines (Das, B., unpublished data). Western blot analysis of the above anti- $\beta$ -reacted membrane extracts with an anti- $\beta$  probe revealed the 51.5-kD band in HPBL as well as tumor cell preparations. This experiment demonstrates that  $\beta$ -H<sup>+</sup>ATPase can be immunoprecipitated from HPBL membrane preparations but the origin of the reactive material must be from the interior of the cell (i.e., mitochondrial) since it is not labeled with biotin (compare lanes B and D of Fig. 5).

**Characterization of the 51.5-kD Lymphocyte-binding Protein.** It



**Figure 4.** Reactivity of anti- $\beta$  with K562 proteins. Proteins from whole cell (lane A) or crude membranes (lane B) were separated by SDS-PAGE and transblotted. Blots were reacted with anti- $\beta$  followed by goat anti-rabbit-alkaline phosphatase and developed by reaction with Nitroblue Tetrazolium chloride and 5-Bromo-4 Chloro-3-indolyl-phosphate *p*-Toluidine (25).



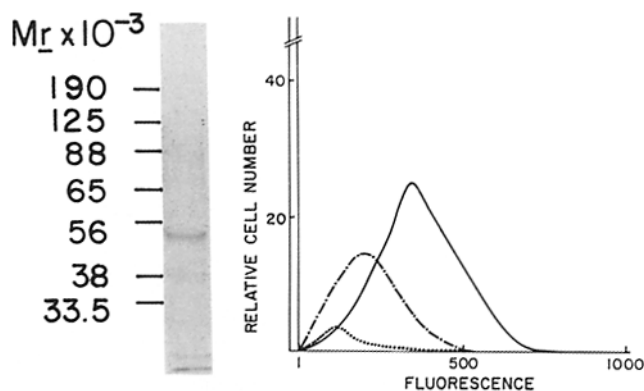
**Figure 5.** Analysis of anti- $\beta$  immunoprecipitates from surface-labeled cells. (Lanes A and B) Western blots of anti- $\beta$  immunoprecipitated proteins from surface biotinylated K562 cells and lymphocytes, respectively. Proteins in these lanes were probed with streptavidin-alkaline phosphatase for the detection of anti- $\beta$ -reactive biotinylated plasma membrane proteins. Note that only K562 cells are positive indicating

the presence of a  $\beta$ -H<sup>+</sup>ATPase-like protein on the surface of K562 cells but not lymphocytes. (Lanes C and D) Western blots of the same anti- $\beta$  immunoprecipitated membrane proteins (lanes A and B) that were probed with anti- $\beta$ , and developed with goat anti-rabbit alkaline phosphatase. Bands represent both cell surface as well as internal anti- $\beta$ -reactive proteins.

is conceivable that a contaminating protein (<5%) in the 51.5-kD isolate (used for amino acid sequence analysis and adhesion studies) is in fact the lymphocyte-binding molecule rather than  $\beta$ -H<sup>+</sup>ATPase. We, therefore, directly investigated whether the lymphocyte binding protein of 51.5 kD from the plasma membranes of K562 tumor cells reacts with anti- $\beta$ . Freshly isolated viable HPBL were first reacted with plasma membrane proteins from cell surface-labeled K562 cells. Subsequently, HPBL were washed to remove unbound tumor proteins and then the HPBL were lysed and the proteins solubilized. The extract from the treated HPBL was reacted with anti- $\beta$ , and the resulting immunoprecipitate subjected to SDS-PAGE, Western blotting, and analyzed for biotin-labeled proteins. The Western blot of this experiment revealed a single biotinylated K562 membrane protein of 51.5 kD indicating that this lymphocyte-binding protein contains the Y311-A331 epitope of  $\beta$ -H<sup>+</sup>ATPase (Fig. 6 left).

Anti- $\beta$  reacts with a peptide sequence of  $\beta$ -H<sup>+</sup>ATP that is involved in the binding of ATP (22). Presumably this antibody would not react with  $\beta$ -H<sup>+</sup>ATPase if the ATP-binding site was occupied, e.g., by combining with a receptor on lymphocytes. To examine this possibility, HPBL were either incubated with purified p51.5 or with medium alone, washed, and then reacted with anti- $\beta$ . These two preparations were further treated with FITC-F(ab')<sub>2</sub> goat anti-rabbit IgG and analyzed by fluorescence-activated flow cytometry. Anti- $\beta$  bound to only those lymphocytes that were first reacted with p51.5 (Fig. 6 right), suggesting that the ATP-binding site of this ligand is not occupied upon reaction with HPBL.

**Effect of Anti- $\beta$  on the Lymphocyte-mediated Cytolytic Activity.** The effect of anti- $\beta$  on cytolytic activity of naive NK and LAK cells was determined. <sup>51</sup>Cr-labeled K562 and A549 cells (NK-sensitive target cells and NK-resistant LAK-sensitive target cells, respectively) were first reacted with anti- $\beta$  and then incubated with effector cells. Significant inhibition in NK-mediated cytolytic activity of naive HPBL as well as LAK cytotoxicity was observed with anti- $\beta$ -treated target cells (Fig. 7, A and B). Inhibition in cytolytic activity was directly proportional to the concentration of anti- $\beta$  used at two different E/T ratios. Inhibition was nearly 100% for NK activity at the lowest E/T cell ratio and highest concentration



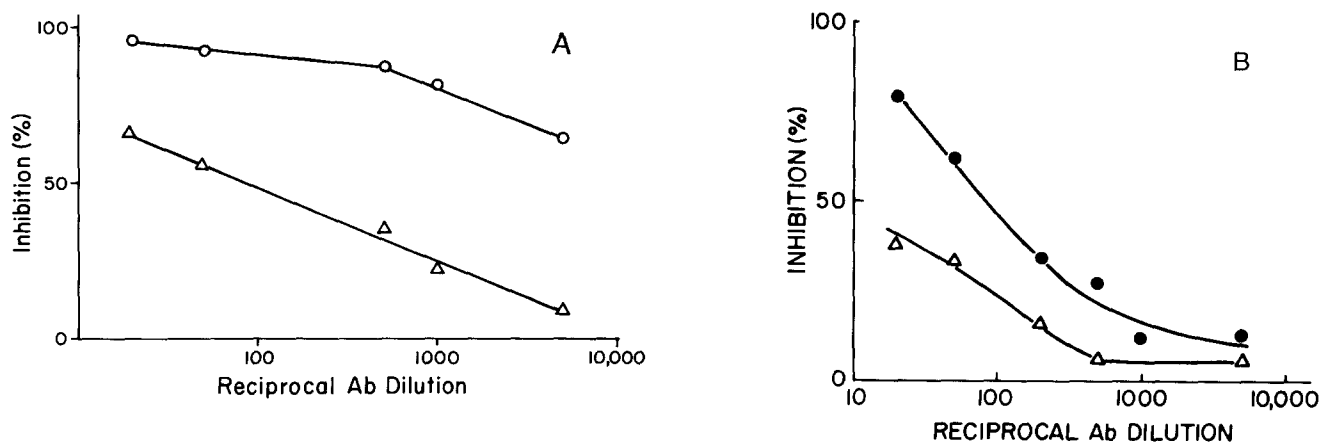
**Figure 6.** (Left) Western blot of the anti- $\beta$  immunoprecipitate of the extract of HPBL that were previously reacted with membrane proteins of the surface biotinylated K562 cells. Briefly, HPBL were reacted with membrane proteins from surface biotinylated K562 cells, washed, and solubilized in 1% Triton X-100. Solubilized proteins from these HPBL were immunoprecipitated with anti- $\beta$  as described in Materials and Methods. Immunoprecipitated proteins were separated by SDS-PAGE and HPBL-bound K562 biotinylated plasma membrane anti- $\beta$ -reactive protein was detected on Western blot by reaction with streptavidin-alkaline phosphatase as described for Fig. 5, lanes A and B. (Right) Flow cytometry analysis of p51.5-treated lymphocytes: interaction with anti- $\beta$ . HPBL ( $2.5 \times 10^6$  cells) were first incubated with 13 (●-●) or 50  $\mu$ g (—) of p51.5, washed with buffer A, incubated with 1:1,000 diluted anti- $\beta$  (4°C, 30 min), and analyzed by flow cytometry. HPBL without p51.5 treatment (.....) served as a control. The mean channel shift (MCS) of fluorescence intensity of HPBL treated with 13 and 50  $\mu$ g p51.5 was 13 and 144, respectively, as compared with the untreated cells. MCS of >10 is considered positive. Representative results from two studies are shown.

of anti- $\beta$  (1:20 dilution). Under similar conditions, LAK cell activity was inhibited by 75%. Undiluted normal rabbit serum and undiluted IgG fraction of rabbit anti-BSA had no effect on NK cell cytotoxicity.

## Discussion

Structural and immunologic investigations demonstrate for the first time a 51.5-kD protein (p51.5) on the plasma membrane of several human tumor cell lines that has homology to  $\beta$ -H<sup>+</sup>ATPase. This protein was not detected on the surface of normal lymphocytes and erythrocytes. Binding and functional studies suggest that p51.5 serves as a ligand in the effector rather than the recognition phase of MHC-independent, cell-mediated cytotoxicity.

$\beta$ -H<sup>+</sup>ATPase is thought to function exclusively as the catalytic subunit of the mitochondrial enzyme responsible for ATP synthesis (21). It was therefore unexpected to obtain data suggesting the expression of  $\beta$ -H<sup>+</sup>ATPase on the plasma membrane of tumor cells. The present study cannot rule out the unlikely possibility of two distinct proteins, one, mitochondrial  $\beta$ -H<sup>+</sup>ATPase and the other, a tumor plasma membrane  $\beta$ -H<sup>+</sup>ATPase-like molecule, both characterized by the same apparent molecular weight (51.5 kD), structural identity at the NH<sub>2</sub>-terminus, and sharing the Y311 to A331 epitope but differing in other regions of the molecule. NH<sub>2</sub>-



**Figure 7.** Effect of anti- $\beta$  treatment of tumor cells on NK- and LAK-mediated cytotoxicity.  $^{51}\text{Cr}$ -labeled target cells were incubated at  $4^\circ\text{C}$  for 30 min with different dilutions of anti- $\beta$  before the addition of fresh unstimulated HPBL (naive NK cells) or IL-2-activated HPBL (LAK cells). Percent cytotoxicity was determined after 3-h incubation by  $^{51}\text{Cr}$  release, and data expressed as percent inhibition relative to a buffer control. (A) Effect of anti- $\beta$  treatment of K562 cells on NK cell-mediated cytotoxicity. (O-O) E/T ratio of 25:1 and ( $\Delta$ - $\Delta$ ) E/T ratio of 50:1. Cytotoxicity of HPBL against K562 cells without anti- $\beta$  (buffer control) was 30% (E/T 25:1) and 59% (E/T 50:1). (B) Effect of anti- $\beta$  treatment of A549 cells on LAK cell-mediated cytotoxicity. ( $\bullet$ - $\bullet$ ) E/T of 25:1 and ( $\Delta$ - $\Delta$ ) E/T of 100:1. Cytotoxicity of LAK cells against A549 cells without anti- $\beta$  (buffer control) was 14% (E/T 25:1) and 35% (E/T 100:1). Fresh unstimulated HPBL had no detectable cytolytic activity against A549 cells (NK-resistant tumor). Incubation of  $^{51}\text{Cr}$ -labeled K562 and A549 cells with anti- $\beta$  alone had no observable effect on cell viability. Data are representative of two experiments.

terminal amino acid sequence analysis of p51.5 indicated 100% homology (at 16 of the 21 positions identified) to  $\beta$ - $\text{H}^+$  ATPase. SDS-PAGE-purified p51.5 contained a single species of protein as deduced by combined quantitative amino acid analysis of the total protein and the  $\text{NH}_2$ -terminal sequenced amino acids. Whereas it is likely that a significant amount of p51.5 was derived from mitochondria (which contaminate crude plasma membrane preparations), immunologic studies clearly revealed that the protein was also expressed on the cell surface. Antibody generated against a peptide in the ATP-binding region of  $\beta$ - $\text{H}^+$  ATPase (anti- $\beta$ ) reacted with viable tumor cells as determined by fluorescence-activated flow cytometry. Since the antibody reacted only with a single protein of 51.5 kD on Western blots of solubilized membrane or whole cell extracts, it is exceedingly unlikely that the immunofluorescence data could be accounted for by cross-reactivity of anti- $\beta$  with other nucleotide-binding proteins. Furthermore, immunoprecipitation studies of surface-labeled cells utilizing anti- $\beta$  indicated immunologic reactivity with a single 51.5-kD protein on the plasma membrane of tumor cells. Taken together, the above studies suggest that plasma membrane-bound p51.5 and mitochondrial-derived p51.5 are identical or nearly so.

It is conceivable that intracellular proteins (e.g.,  $\beta$ - $\text{H}^+$  ATPase) may be released from a small percentage of dead tumor cells in tissue culture and that the protein may bind to the surface of the remaining viable cells. The latter proposal cannot, however, explain the substantial amount of anti- $\beta$ -reactive protein detected on the plasma membrane of tumor cells relative to normal cells. Comparison of surface-labeled p51.5 to whole cell extract-labeled p51.5 by semi-quantitative ELISA analysis indicated that  $\sim 40\%$  of the total cellular anti- $\beta$ -reactive protein in K562 cells is associated with the exterior of the plasma membrane (Das, B., unpublished data). Since only 1% of the tumor cells was nonviable, it is doubtful

that they could contribute the amount of surface p51.5 as determined in the above estimates. Moreover, immunoprecipitation studies of surface-labeled tumor cells indicate that p51.5 could not be removed from the membranes by treatment with 0.5 M NaCl and/or 1 mM EDTA (Das, B., unpublished data). These results further rule out the possibility that  $\beta$ - $\text{H}^+$  ATPase is bound to the cell surface by nonspecific interaction, i.e., binding of p51.5 from dead cells. Further studies are in progress to establish the mode of attachment of p51.5 to the plasma membrane.

Data presented in this report demonstrate that cell surface  $\beta$ - $\text{H}^+$  ATPase or a molecule that is remarkably similar acts as a functional ligand in lymphocyte-mediated cytotoxicity. Purified p51.5, in contrast to another tumor membrane protein, bound to human lymphocytes, suggesting a specific receptor-ligand interaction. Labeled p51.5 from surface biotinylated K562 cells could be immunoprecipitated from HPBL treated with crude tumor membrane extracts by specific antibody to  $\beta$ - $\text{H}^+$  ATPase. p51.5-treated HPBL were blocked in their ability to mediate NK cytotoxicity presumably because of inability of the lymphocyte receptor to engage the p51.5 ligand on the plasma membrane of K562 target cells. Furthermore, anti- $\beta$  was exceedingly effective in blocking naive NK cytotoxicity ( $>95\%$  inhibition) and highly effective (75% inhibition) in preventing LAK cytotoxicity. We note that the antibody used in the latter studies was whole IgG. It is well known that NK cells can mediate lysis of NK-resistant target cells if IgG is specifically bound to the cell surface (antibody-dependent, cell-mediated cytotoxicity, ADCC) (3, 6). Consequently, one may infer that the ADCC pathway uses p51.5 as a ligand since inhibition of cytotoxicity, rather than enhancement, was observed with the anti- $\beta$  treatment of K562 and A549 cells. These data are consistent with the hypothesis that  $\beta$ - $\text{H}^+$  ATPase serves as a ligand in the effector rather than the recognition phase of a common final lytic pathway

used in NK, ADCC, and LAK cytotoxicity, since recognition is likely due to different molecular species in cells mediating the latter activities.

It is interesting to note that anti- $\beta$  was relatively ineffective in blocking cytotoxicity mediated by the YT cell line (<25% inhibition). Since YT-induced tumor destruction is mediated to a large extent by lytic granules (31), p51.5 is probably not utilized as a ligand in the granule exocytosis pathway. In this regard, Valiante and Trinchieri (32) have identified a signal transduction surface molecule on cytolytic lymphocytes that does not mediate release of granule enzymes.

Experimental evidence suggested that the ATP-binding domain and the lymphocyte-receptor binding site of p51.5 are located in different regions of the molecule. Flow cytometry analysis indicated that antibody directed against an epitope in the ATP-binding domain (anti- $\beta$ ) of  $\beta$ -H<sup>+</sup> ATPase could react with the solubilized p51.5 which was bound to the surface of lymphocytes. This finding suggests that the catalytic site of p51.5 was not blocked upon interaction of the molecule with its lymphocyte receptor. We infer that inhibition of cytotoxicity by anti- $\beta$  is not due to blockage of receptor-ligand engagement but rather is due to inhibition of the catalytic function of the ligand. Of course, it is possible that p51.5 may function in cytotoxicity in a different manner from its usual catalytic role. Indeed the  $\alpha$  subunit of the mitochondrial H<sup>+</sup>-transporting ATP synthase has been found in peroxisomes and was suggested to function as a molecular chaperon in both organelles, besides its usual role in ATP synthesis in mitochondria (33).

Mitochondrial H<sup>+</sup> transporting ATP synthase is a multimeric enzyme that is composed of two functional domains; "F<sub>0</sub>," the transmembrane H<sup>+</sup> channel, and "F<sub>1</sub>," the catalytic site for ATP synthesis (21, 34). The  $\beta$  subunit that contains the ATP-binding domain (catalytic site) of the enzyme is a protein encoded by a nuclear gene. In normal cells it is synthesized in the cytoplasm and translocated to the inner membrane of mitochondria where it associates as a nontransmembrane protein with other subunits of the F<sub>1</sub> complex (21, 34–36). Whereas altered expression of such a molecule on tumor cells was unexpected, previous studies (37–40) have shown a decrease in ATP synthase activity and a reduction in the content of F<sub>1</sub> components in mitochondria isolated from neoplastic cells and from cells of regenerating tissues. Significant alterations in the content of F<sub>0</sub> were not observed in these studies (38–40). Postulated causes of alterations in the F<sub>1</sub> complex of cancer cells were decreased synthesis of F<sub>1</sub> proteins, defective mitochondrial uptake of the proteins, and/or abnormal translocation of F<sub>1</sub> subunits (38–40). Data in this report support the latter hypothesis. Clearly, further studies are necessary to determine if p51.5 expression on the plasma membrane occurs in a wider variety of tumors and

normal tissues compared with the limited number examined in the present investigation.

The interaction of plasma membrane  $\beta$ -H<sup>+</sup> ATPase with a putative lymphocyte receptor may provide insights into previous observations regarding the role of ATP in tumor cell destruction. Addition of ATP to culture of tumor cell lines is known to induce membrane depolarization, changes in permeability, and eventual lysis of a variety of transformed cells (14–18, 41, 42). Nontransformed cells, with the exception of mast cells (43) and macrophages (44), were unaffected by exogenous ATP. The cytolytic effect of ATP on these cells was specific in that other purine or pyrimidine nucleotides did not mimic the response. Rozengurt et al. (15, 16) suggested that ATP facilitates the formation of aqueous ionic pores resulting in imbalance in ionic homeostasis, swelling, and eventual lysis of cells.

Based on the above observations, these investigators suggested that lymphocyte-induced tumor destruction may use eATP as a mediator in a lytic pathway (14, 41, 42). Direct evidence for this proposal, although quite limited, is nevertheless compelling. Henney (19), while investigating possible markers of lymphocyte-induced target cell death, observed an increase in eATP within minutes of effector-tumor cell interaction. The amount of ATP in the supernatant was significantly greater than the amount present in target cells alone. Although contribution of ATP from effector cells due to leakiness was suggested, lymphocyte permeability alterations using other markers were not observed (19). Filippini et al. (45) demonstrated an increase in lymphocyte plasma membrane-associated ATP after stimulation of T cells by anti-CD3 antibody or by Con A. The latter investigators suggested that ATP may be generated at the cell surface of the lymphocyte upon activation by the target cell. Our results demonstrating the presence of  $\beta$ -H<sup>+</sup> ATPase on the surface of neoplastic cells, and the inhibitory effect of both soluble p51.5 and anti- $\beta$  suggest the involvement of an energy-using system of tumor membrane origin in lymphocyte-mediated cytotoxicity since the  $\beta$  subunit can in fact, under appropriate conditions, hydrolyze ATP to ADP (46–48). Whereas the precise mechanism by which  $\beta$ -H<sup>+</sup> ATPase functions in lymphocyte-mediated cytotoxicity remains to be determined, it is tempting to speculate that p51.5, upon combination with its lymphocyte receptor(s), may provide energy for transport of H<sup>+</sup> across the plasma membrane into the tumor cell resulting in cytolysis by osmotic shock. Target cell membrane depolarization and osmotic swelling are well-known phenomena in lymphocyte-mediated destruction of tumors (11, 13). The orientation of  $\beta$ -H<sup>+</sup> ATPase on the plasma membrane with its extracellular ATP binding domain would permit such activity.

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