An Acid Protease Secreted by Transformed Cells Interferes with Antigen Processing

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Abstract. The major excreted protein of malignantly transformed mouse fibroblasts (MEP), which is the precursor to lysosomal cathepsin L, was used to study the effect of exogenous acid proteases on antigen processing. When MEP and native pigeon cytochrome c were added to Chinese hamster ovary (CHO) cells expressing transfected major histocompatibility complex class II gene products, the antigen-specific T-cell hybridoma 2B4 did not respond to the antigen, MEP appears to destroy the antigen in an acid compartment of the presenting cell because: (a) MEP is only active as a protease under acid conditions; (b) mannose 6-phosphate inhibited the internalization of MEP and blocked its effect on antigen processing; (c) the destruction required the simultaneous entry of the antigen and MEP into the cells; and (d) cytochrome c fragment 66–104 which does not need to be processed stimulated 2B4 in the presence of MEP. These results support the hypothesis that antigen processing requires internalization of the antigen into an acidic compartment, and they provide a new model for the investigation of the contribution of acid proteases to the reduced immunocompetence of tumor-bearing animals.

FOREIGN antigens bound to appropriate Major Histocompatibility Complex (MHC) class II molecules on the cell surface of antigen-presenting cells (APC) are usually required for the activation of CD4+ T cells (Babcock et al., 1986; Unanue and Allen, 1987). Antigen recognition by T cells is often specific for determinants formed by peptide fragments of soluble protein antigens rather than determinants on the native structure of the antigen (Ziegler and Unanue, 1982; Allen and Unanue, 1984; Chestnut et al., 1982; Shimonkevitz et al., 1983, 1984; Streicher et al., 1984; Kovac and Schwartz, 1985; Ashwell et al., 1986). Current models hypothesize that the APCs generate these peptide fragments by internalizing the native antigen, cleaving it in an acidic compartment, and returning the fragments to the cell surface (Ziegler and Unanue, 1982; Allen and Unanue, 1984; Chestnut et al., 1982; Streicher et al., 1984; Kovac and Schwartz, 1985). This series of events is referred to as antigen processing. Although certain acid proteases are believed to mediate antigen processing (Streicher et al., 1984; Buus and Werdelin, 1986), the mechanism and regulation of this process are not well understood.

Malignantly transformed cells secrete a large number of biologically active materials, including both neutral proteases (Unkeless et al., 1973; Salo et al., 1983; and Matrisian et al., 1986) and acid proteases such as cathepsins B, D, and L (Poole et al., 1978; Sloane et al., 1981; Mort et al., 1981; Gal and Gottesman, 1986b; Capony et al., 1987). Proposed functions of the neutral proteases, such as plasminogen activator, include alteration of the extracellular matrix surrounding tumor cells, enhancement of local invasiveness, and involvement in metastasis. The function of the acid proteases is unclear, however, because they would not be expected to be active under the nearly neutral pH conditions of the extracellular space and plasma.

We have previously reported the increased synthesis and secretion by transformed mouse fibroblasts of a 39,000-D molecular weight protein, termed MEP (for major excreted protein) (Gottesman, 1978; Gottesman and Sobel, 1980). This polypeptide is an enzymatically active precursor to a cysteine protease that has recently been found to be mouse cathepsin L (Gal and Gottesman, 1986a, 1987). MEP carries the lysosomal mannose-6-phosphate (M6P) recognition unit (Sahagian and Gottesman, 1983; Gal et al., 1985) and could, therefore, be internalized via M6P receptors into an acidic compartment where it would be active. The aim of our study was to determine whether the secreted form of MEP could alter the immune response of CD4+ T cells. We found that the acid protease MEP when internalized along with the antigen, pigeon cytochrome c, interferes with antigen processing by destroying the antigenic determinant.

1. Abbreviations used in this paper: APC, antigen-presenting cells; EMEM, Eagle's minimum essential medium; MEP, major excreted protein; MHC, major histocompatibility complex; M6P, mannose-6-phosphate.
Materials and Methods

Cell Lines

The derivation and specificity of pigeon cytochrome c–specific, E6×E4-restricted T-cell hybridoma 2B4 were previously described (Hedrick et al., 1982). The lambda repressor-specific, E4×E4-restricted T-cell hybridoma 8I was kindly provided by Dr. Malcolm Gefter (Massachusetts Institute of Technology, Boston, MA), and the specificity of the hybridoma was previously published (Guillet et al., 1987). The isolation of the parental cell WTB from a CHO cell line has been previously described (Thompson and Baker, 1973), and WTB was kindly provided by Dr. April Robbins (National Institutes of Health, Bethesda, MD). The IL-2 dependent cell line CTLL was derived by Baker et al. (1979) and was kindly provided by Dr. Ethan Shevach (National Institutes of Health, Bethesda, MD).

Cell Culture

Selective medium for growth of transfectant WE2A4 consisted of Eagle's minimum essential medium (EMEM) (Biofluids, Rockville, MD) containing 5% FCS, 2 mM glutamine, 0.1 mM nonessential amino acids, antibiotics, and MxH (6 μg/mL mycoplgonic acid, 250 μg/mL xanthine, and 15 μg/mL hypoxanthine). WE2A4 was grown at 34°C, and the other cells were maintained at 37°C. LK35.2, 8I, and CTLL were cultured in RPMI-1640 (Biofluids) containing the above supplements plus 50 μM 2-mercaptoethanol and 10% FCS. 2B4 was grown in 50% RPMI-1640:50% Click's medium (Biofluids) containing the same supplements as complete RPMI-1640.

Antigens

Pigeon cytochrome c was purchased from Sigma Chemical Co. (St. Louis, MO). Cyaogen bromide cleavage fragments of cytochrome c were prepared as previously described (Corradin and Harbury, 1970). The apo form of cytochrome c was prepared by ethyl acetate extraction under acidic conditions as described (Savage and Fontana, 1977). The purity of the cytochrome c peptides and apo form was verified by reverse phase HPLC on a C-18 column (Vydac, Southburo, MA) and an ion exchange column (Custom LC Inc., Houston, TX), respectively. Lambda repressor 1–102 was kindly provided by Dr. Malcolm Gefter (Massachusetts Institute of Technology, Boston, MA).

Preparation of MEP

HPLC purification of MEP was as previously described (Gal and Gottesman, 1986a). [35S]Methionine labeled MEP was prepared by collection of medium from KNIH cells. Radio-labeled MEP was precipitated from conditioned medium by adding ammonium sulfate to 80% saturation followed by centrifugation at 17,000 g. The pellet was dissolved in 10 mM sodium phosphate, pH 6.5, 150 mM NaCl and dialyzed against four changes of the same buffer.

Stimulation of T Cells and the IL-2 Assay

The transfectants were extensively washed in EMEM to remove mycoplgonic acid before use. Cells were plated in 96-well flat-bottom microtiter plates (Costar, Cambridge, MA) with 5 × 104 APCs, 3 × 105 T cells, and various doses of antigen in a total volume of 200 μl. In some experiments, 10 mM M6P (Sigma Chemical Co.) was added to the wells. After 24 h, 100 μl of culture supernatant was removed. Supernatants were assayed for the presence of IL-2 by incubation of the IL-2 dependent cell line CTLL with two-fold serial dilutions of the supernatant. After 16 h, the cells were pulsed with 1 μCi of [H]thymidine (6.7 Ci/mmol, New England Nuclear, Boston, MA) in 0.5 ml after another 3 h. The cells were washed three times with ice and washed. The cells were resuspended in 0.4 ml PBS containing 1% FCS and 0.02% NaN3. The fluorescence intensity of viable cells was measured by a Becton Dickinson FACS Analyzer (Mountain View, CA) equipped with a mercury-arc lamp and appropriate excitation filters. Cell volume gates were set to exclude dead cells and cell clumps. Data were collected on 10,000 cells with log amplification and analyzed by a Hewlett-Packard Series 200 Computer (Purdord, OR).

Uptake and Processing of MEP

WE2A4 cells (2 × 106/60-mm dish) were plated 48 h before the uptake experiment. The cells were treated for 10 min at room temperature in EMEM containing 50 mM Hepes, pH 7.4, and 10 mM M6P to remove any ligand bound to M6P receptors. Cells were then washed eight times with EMEM containing 50 mM Hepes. [35S]Methionine labeled KNIH conditioned medium containing MEP (5 × 106 cpm/dish) was incubated with the washed WE2A4 cells for varying lengths of time in the presence or absence of 10 mM M6P. At the end of the appropriate time period, cells were washed four times in PBS and lysed in immunoprecipitation buffer A (0.154 M NaCl, 0.05 M Tris-HCl, pH 7.4, 0.5% Nonidet P-40, and 0.05% SDS). Lysates were immunoprecipitated and analyzed by fluorography of 12% acrylamide gels as previously described (Gottesman and Cabral, 1981), but without linear polyacrylamide or glycerol.

Results

Incubation of MEP In Vitro with Pigeon Cytochrome c

MEP is a very broad spectrum cysteine protease that cleaves proteins at multiple sites with pH optima between 3 and 6 (Gal and Gottesman, 1986a; Mason et al., 1987). The smallest antigenic peptide of cytochrome c fully recognized by the T-cell hybridoma 2B4 is a peptide corresponding to residues 95–103 of the native molecule (Schwartz et al., 1985). Extensive mapping of the antigenic determinant demonstrated that only cleavage within this region renders...
the antigen almost completely nonstimulatory. We examined the possibility that MEP could affect the antigenicity of pigeon cytochrome c by incubating cytochrome c with MEP under acidic conditions. The preparations were neutralized and tested for their ability to stimulate 2B4 to secrete interleukin-2 (IL-2) in the presence of APCs (LK35.2), a B-cell hybridoma. As shown in Fig. 1 A, preincubation of intact antigen with MEP caused a decrease in the response to 2B4 which was dependent upon the length of time of preincubation. Analogously, the preincubation of MEP with the CNBr peptide fragment 66–104 of pigeon cytochrome c abrogated the response of 2B4 (Fig. 1 B). This fragment is a substrate for MEP in vitro (data not shown). The loss of responsiveness was not merely due to the acidic conditions of the preincubation, as the stimulatory capability of 66–104 was not affected by the acidic buffer. In addition, boiled MEP, which lacks enzymatic activity, was only slightly inhibitory. Most importantly, the effect of MEP was completely reversed by the cysteine protease inhibitor, leupeptin, which blocks the enzymatic activity of MEP (Gal and Gottesman, 1986a). MEP had no effect upon the proliferation of CTLL, an IL-2 dependent cell line, in the presence of our standard IL-2 preparation (data not shown) indicating that the decrease in the 2B4 response was not due to cleavage of IL-2 by MEP carried over into the assay. These results indicate that acid-activated MEP can destroy the antigenic determinant of pigeon cytochrome c recognized by 2B4.

**Mep Affects Antigen Processing**

We next examined whether MEP could affect antigen processing and/or presentation. MEP can be internalized by cells via M6P receptors. Chinese hamster ovary (CHO) cells express a high number of these receptors upon their cell surface (see below), and, thus, we chose these cells transfected with the genes encoding E\textsubscript{a}:E\textsubscript{b} molecules (named WE2A4) as APCs. A representative experiment is shown in Fig. 2. In the presence of 1.3 μM MEP, the response to the apo form of cytochrome c, which requires processing for presentation (Kovac and Schwartz, 1985), was inhibited by at least 30-fold (Fig. 2 A). The inhibitory effect of MEP upon the T-cell stimulation was reversed by 10 mM M6P suggesting that uptake via the M6P receptors was essential for the effect. The inhibition by MEP depended upon the concentration of MEP and was routinely observed when MEP was added to the cultures at a concentration of at least 0.5 μM (data not shown). The presence of 1.3 μM MEP in the cultures consistently resulted in a decrease in the amount of IL-2 secreted by 2B4, and the degree of inhibition was 48, 63, and 84% (average 65%) in three experiments. In all cases, the addition of M6P reversed this inhibition. Similar results were found when the B-cell hybridoma LK35.2 was used as the presenting cell, although since these cells have fewer M6P receptors, the extent of inhibition by MEP was less (data not shown).

To determine whether there were any nonspecific effects of MEP on APC or T-cell function that might account for this inhibition, several control experiments were performed. First, the possible effect of MEP upon the expression of surface molecules necessary for CD4+ T cell responses was examined. Incubation of the APCs or 2B4 with MEP for 24 h did not cause a diminution in the surface expression of E\textsubscript{a}:E\textsubscript{b} molecules or T-cell receptors, respectively, as determined by immunofluorescence staining and microfluorimetric analysis (data not shown). Second, the response to peptide 66–104 was measured in the presence of MEP. The T-cell recognition of this fragment is not affected by any known treatments that block antigen processing, because it does not need to be internalized prior to presentation to the T cells (Kovac and Schwartz, 1985). As shown in Fig. 2 B, MEP did not interfere with the T-cell response to peptide 66–104 indicating that antigen presentation resulted predominantly from the association of extracellular peptide and cell surface MHC class II molecules on the APC. Thus, we conclude that MEP is inhibiting T-cell activation by interfering with the antigen processing phase of the response.

Finally, we used another antigen system which also involves internalization of the antigen by the APC, to determine whether MEP exerts its effect by influencing the intracellular processing machinery or only the antigen. Lambda repressor 1–102 requires processing to elicit a response by
the T-cell hybridoma 81 (Guillet et al., 1987). In contrast to the results with cytochrome c, the preincubation of lambda repressor with MEP under acidic conditions did not abrogate the T-cell response (data not shown). Thus, the critical antigenic determinant, residues 12-24, recognized by 81 is not destroyed by MEP. As shown in Fig. 3, MEP at 0.7 μM had no effect upon the ability of WE2A4 to stimulate 81 in the presence of various concentrations of lambda repressor. MEP at 1.3 μM was also not inhibitory (data not shown). Thus, we conclude that MEP is selectively interfering with the antigen processing of pigeon cytochrome c by destroying the antigenic determinant.

**MEP Is Internalized via M6P Receptors on the APCs**

We hypothesized that interference with antigen presentation by MEP depended on the presence of MEP and antigen in an acidic intracellular compartment where MEP could become enzymatically active. To support this idea, we tested the ability of WE2A4 cells to process MEP to lower molecular weight forms normally found in lysosomes (Gal et al., 1985). As shown in Fig. 4, radiolabeled MEP binds to WE2A4 cells and is gradually processed over 1-5 h. This processing can be blocked by incubation with M6P. Similar results were found with the parental CHO line WTB (data not shown). Exogenous MEP is processed to two lower molecular weight forms of 29,000 and 20,000 D. The 29,000-D form appeared first at 1 h, and the smallest form was observed subsequently at 3 h. Since these lower molecular weight forms are never seen after incubation of MEP at neutral pH, and they are characteristic of the intracellular processing of MEP in an acidic compartment, we conclude that MEP has been internalized via M6P receptors on the APCs.

**Temporal Requirement for the Action of MEP**

The above results suggest that MEP interferes with antigen processing by digesting the antigen in an acidic intracellular compartment. The temporal requirement for the inhibition of antigen processing by MEP was investigated. WE2A4 was pulsed with 4 mM intact cytochrome c and fixed with paraformaldehyde before the stimulation of 2B4. The antigen pulse allows the CHO cells to process the intact molecule into immunogenic peptides, and the fixation prevents any further internalization and processing by the cells. Data from a representative experiment are presented in Fig. 5. When MEP was added after the antigen pulse and fixation, there was no effect upon the response of 2B4 suggesting that MEP had to be internalized by the APCs to become active. However, when MEP was present during the antigen pulse, the amount of IL-2 secreted by 2B4 decreased by ~85%. Again, the inhibitory effect of MEP was reversed by M6P. Interestingly, when the APCs were incubated with MEP before the antigen pulse, there was a normal response by 2B4. Thus, a decrease in the level of the T-cell response was only observed when MEP and intact cytochrome c were incubated simultaneously with the APCs.

**Discussion**

These results indicate that MEP, an acid protease secreted by transformed cells, can interfere with antigen processing. MEP inhibited the ability of LK35.2 cells and of CHO cells transfected with MHC class II genes to process both native and apo pigeon cytochrome c resulting in a decreased amount of IL-2 secreted by an antigen-specific CD4+ T-cell hybridoma. This inhibition depended upon the concentration of MEP but was not observed in cultures containing the CNBr fragment 66-104 of cytochrome c which does not require internalization and intracellular processing to elicit a T-cell response (Kovac and Schwartz, 1985). By contrast, both the native form and the peptide fragment of cytochrome c lost...
their antigenicity if they were incubated under acidic conditions with MEP before stimulation of the T cells, indicating that MEP could destroy the antigenicity of all forms of cytochrome c under conditions where it is active as a protease. MEP did not diminish the expression of T-cell receptors on the T-cell hybridoma nor MHC class II molecules on the APCs. MEP also did not affect the proliferation of CTLL in the presence of our standard IL-2 preparation demonstrating that a decrease in the T-cell response was not merely due to cleavage of IL-2.

MEP is the precursor to a lysosomal protease that requires acidic conditions for its enzymatic activity, as do the lower molecular weight forms of MEP that are produced by autocatalysis (Gal and Gottesman, 1986a, b; Mason et al., 1987). Our results strongly indicate that MEP must be internalized by the APC via M6P receptors to cause an inhibitory effect upon T-cell stimulation for the following reasons: (a) M6P blocked the inhibitory effect of MEP; (b) the T-cell response to antigenic peptide fragment was normal in the presence of MEP; and the peptide does not require internalization and processing by the APC; and (c) when the APCs were first pulsed with antigen and then fixed, the presence of MEP during the T-cell stimulation had no effect.

Our findings suggest that acid-activated MEP is acting directly upon the antigen itself inside the APC. At least part of the internalized MEP and cytochrome c are probably transported to the same intracellular acidic compartment, where MEP is activated and cleaves cytochrome c to non-stimulatory peptides. The time course for the conversion of precursor MEP into the mature forms (Fig. 4) parallels that for antigen processing (Chestnut et al., 1982; Ziegler and Unanue, 1982). In both cases, there is a lag period of ~1 h. By this model, the inhibitory effect of MEP should depend on the existence of a cleavage site within the antigenic determinant recognized by the T cell, as was shown for cytochrome c. Because of the broad spectrum of activity of MEP (Gal and Gottesman, 1986a), a large number of potential antigenic determinants would be expected to be substrates for this protease. In contrast, the T-cell response to lambda repressor was not abrogated by MEP, and this antigen also requires internalization and processing by the APCs. The lack of a reduced T-cell response after preincubation of lambda repressor with MEP under acidic conditions indicates that lambda repressor does not contain a MEP cleavage site in the peptide fragment 12–24, which is the smallest peptide recognized by the T-cell hybridoma (Guillet et al., 1987).

An alternative possibility to direct antigen cleavage by MEP is that MEP may block or destroy the activity of antigen processing enzymes. If this were the case, then the processing mechanism for lambda repressor must be different from that of cytochrome c, as the response to lambda repressor was unaffected by MEP. Arguing against this possibility is the temporal requirement of the inhibition in that MEP must be added simultaneously with the antigen to the APCs to see an effect. When MEP is added before the antigen, MEP has no effect upon the T-cell response. If MEP were destroying the processing enzymes, one might expect interference with processing when MEP was added prior to the antigen unless the inactivated processing enzymes were very rapidly replaced. This observation also indicates that the presence of internalized MEP within a given compartment is transient, i.e., MEP did not accumulate in the compartment where it could act upon the antigen. In fact, this finding argues that the antigen and MEP must be present in the same vesicles in order for MEP to interfere with antigen processing. This conclusion is consistent with the previous observation that ligands labeled with two different conjugates, when added sequentially to cells, do not enter the same vesicles (Hanover et al., 1984).

Previous studies have demonstrated that lysosomotropic reagents, such as NH4Cl, chloroquine and monensin, which elevate the intracellular pH can block antigen processing (Ziegler and Unanue, 1982; Chestnut et al., 1982; Allen and Unanue, 1984; Kovac and Schwartz, 1985). The current model suggesting that antigens are processed within intracellular acid compartments is based on these data. However, these pharmacologic reagents have numerous secondary effects upon cells, including alteration of transport pathways and inhibition of vesicle fusion (Seglen, 1983). By contrast, MEP should not influence the trafficking of cytochrome c within the APC, and, therefore, interferes with antigen processing in the absence of these secondary effects. Thus, our results provide further evidence which substantiates the idea that extracellular antigens are endocytosed and processed within an intracellular acidic compartment of the APCs.

The T-cell response to cytochrome c peptide fragment 66–104 was normal in cultures containing MEP. Although the peptide could be internalized by the APCs where acid-activated MEP should destroy it, the lack of any effect of MEP on the dose–response curve (Fig. 2 B) indicates that presentation of this peptide involves predominantly the association of extracellular peptide and cell surface MHC class II molecules. This finding agrees with the inability of chloroquine to inhibit the T-cell response to peptide 66–104 (Kovac and Schwartz, 1985; McCoy, K., and R. H. Schwartz, unpublished observation).

The consequence of treating APCs with MEP or chloroquine is identical; interference with antigen processing results in a lower T-cell response. However, the mechanisms whereby these treatments influence antigen processing differ greatly. Chloroquine is thought to block antigen processing by inhibiting the enzymatic activity of the cathepsins and preventing the exposure of the antigenic determinant, whereas MEP, a cathepsin, has the opposite effect and blocks antigen processing by cleaving the antigenic determinant. The ability of active acid proteases both to generate antigenic peptides and to destroy them appears to be a paradox. However, it has been estimated that <1.0% of native antigen molecules are converted to immunogenic peptides, and thus the bulk of internalized antigen is normally degraded into nonstimulatory fragments (Shevach, 1984; Unanue, 1984). Furthermore, the entry of MEP into the APC via M6P receptor-mediated endocytosis probably increases the local concentration of this active enzyme within acidic compartments beyond its normal levels. Alternatively, the peptide bond specificity of MEP may be different from that of normal processing protease(s), thereby resulting in a greater amount of cytochrome c being degraded to antigenically inactive forms. It remains to be determined whether MEP can influence the processing of endogenously synthesized antigens.

It is possible that our findings may represent a more general phenomenon. At least two other precursor lysosomal enzymes have been found in the secretions of tumor cells,
i.e., cathepsins B and D (Poole et al., 1978; Sloane et al., 1981; Capony et al., 1987). These enzymes have different specificities and thus could degrade other immunogenic peptides. Our findings may have implications in vivo, because large amounts of acidic proteases are secreted by some tumors. For MEP (cathepsin L), levels used in these studies to interfere with antigen processing (<13 ng/mL) are similar to concentrations of MEP found in medium conditioned by tumor cells (1 µM), and hence could be physiologically significant. Thus, the cathepsins could contribute to the depressed immunocompetence exhibited by tumor-bearing animals.

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