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Immunology

Serum proteases alter the antigenicity of peptides presented by class I major histocompatibility complex molecules

(antigen presentation/cytotoxic T lymphocytes/immunotherapy/vaccine)

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ABSTRACT **Any effect of serum on the antigenicity of peptides is potentially relevant to their use as immunogens *in vivo*. Here we demonstrate that serum contains distinct proteases that can increase or decrease the antigenicity of peptides. By using a functional assay, we show that a serum component other than β_2-microglobulin enhances the presentation of ovalbumin peptides produced by cyanogen bromide cleavage. Three features of this serum activity implicate proteolysis: it is temperature dependent, it results in increased antigenicity in a low molecular weight peptide fraction, and it is inhibited by the protease inhibitor leupeptin. Conversely, presentation of the synthetic peptide OVA-(257–264) is inhibited by serum. This inhibition is unaffected by leupeptin but is blocked by bestatin, a protease inhibitor with distinct substrate specificities. Implications for peptide-based vaccine design and immunotherapy are discussed.**

Cytotoxic T lymphocytes (CTLs) recognize peptide antigens bound to class I major histocompatibility complex (MHC) molecules on the surface of target cells. The peptide binding groove of the MHC class I molecule is stabilized by the interaction of a transmembrane heavy chain encoded by the MHC and a noncovalently associated nontransmembrane protein, β_2-microglobulin. Cell surface MHC class I molecules are largely unreceptive to exogenous peptides (1–4). They typically present only endogenously synthesized peptides. These peptides are encountered during the biosynthetic assembly of the MHC class I complex in intercellular compartments accessible to endogenously synthesized antigen, but separate from those compartments containing internalized exogenous antigen (5). This segregation selectively targets MHC class I-restricted CTLs to pathologically affected cells (i.e., cells expressing abnormal proteins). Similarly, this segregated processing pathway and the inaccessibility of cell surface MHC class I molecules to peptide may explain why attempts to prime CTL responses by using exogenous peptide have generally failed (6, 7).

Serum facilitates the presentation of peptide in MHC class I-restricted responses. This effect has been attributed to the presence of free β_2-microglobulin, which significantly increases the receptivity of cell surface MHC class I complexes for exogenous peptide (1–4). We recently noted a serum enhancement of MHC class I-restricted peptide presentation not entirely accounted for by the concentration of β_2-microglobulin. Here we characterize the basis for this effect. We show that serum contains a variety of proteolytic activities that can increase or decrease peptide antigenicity for MHC class I-restricted T-cell activation. This differential effect is dependent on both the proteolytic specificity of serum components and the nature of the peptide. Implications for peptide-based vaccine design and immunotherapy are discussed.

MATERIALS AND METHODS

Reagents. Chicken ovalbumin (OVA) was purchased from Sigma. CNBr cleavage of OVA was preformed as described (8). The peptide corresponding to amino acids 257–264 [OVA-(257–264)] was synthesized at the molecular biology core facility of the Dana–Farber Cancer Institute. Purified human β_2-microglobulin, leupeptin, and bestatin were purchased from Sigma.

Cell Lines. The RF33.70 (anti-OVA + K^b) (9) and DO.11.10 (anti-OVA + $IA^{d/b}$) (10) T–T hybrids have been described, as has the C57BL/6 T-cell lymphoma EL4 (11).

Treatment of Antigen-Presenting Cells (APCs). APCs were passaged in medium supplemented with 10% (vol/vol) calf serum until 24 h before antigen pulsing, at which time they were washed and cultured in serum-free complete Opti-MEM (GIBCO). For APC pulsing, APCs were incubated in serum-free complete Opti-MEM with or without antigen and the indicated amounts of human β_2-microglobulin, mouse serum, and/or protease inhibitor for 3 h at 37°C as indicated. APCs were then washed extensively and fixed with paraformaldehyde as described (12).

Pretreatment of CNBr-Cleaved OVA with Serum. CNBr-cleaved OVA (60 μg/ml) was incubated in complete Opti-MEM with or without the indicated concentration of mouse serum for 3 h at the indicated temperature. Subsequently, 2 ml of each sample was centrifuged through a Centricon-3 filter (Amicon) at 4°C. The indicated volume of filtrate was incubated with 10^6 EL4 cells in serum-free complete Opti-MEM with β_2-microglobulin (10 μg/ml) in a combined volume of 1 ml for 3 h at 37°C. EL4 cells were then washed and fixed with paraformaldehyde as described.

Hybridoma Cultures and Lymphokine Assays. T–T hybridoma cultures were prepared as described (1, 9). Briefly, 10^5 T–T hybrids were cultured with the indicated number of paraformaldehyde-fixed APCs. After 18 h of incubation at 37°C, 100 μl of supernatant was removed and subjected to freeze–thaw cycles. The lymphokine (interleukin 2) content in T-cell hybridoma culture supernatants was measured with a quantitative bioassay with HT2 cells (13) as described (9, 14). In the absence of antigenic stimulation, the control was usually $<1 \times 10^3$ cpm.

RESULTS

Serum Augments Presentation of CNBr-Cleaved OVA but Inhibits Presentation of the Short Peptide OVA-(257–264). Free β_2-microglobulin facilitates the association of exoge-

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Abbreviations: MHC, major histocompatibility complex; CTL, cytotoxic T lymphocyte; OVA, ovalbumin; APC, antigen presenting cell.

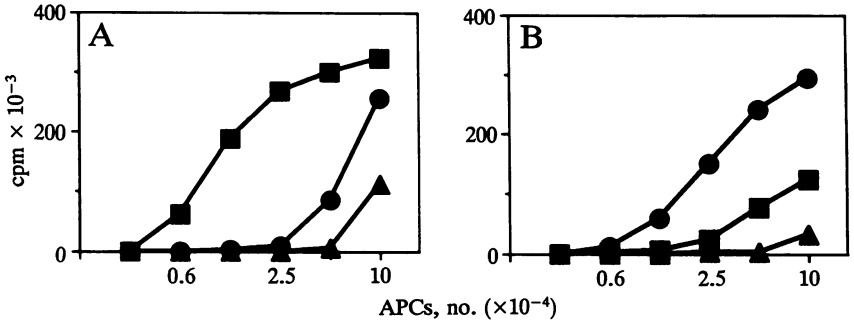

FIG. 1. Serum augments presentation of CNBr-cleaved OVA with MHC class I K^b molecules but inhibits presentation of the synthetic peptide OVA-(257–264). (*A*) EL4 cells (10^6 cells per ml) were incubated in complete Opti-MEM (serum free) for 3 h at 37°C with CNBr-cleaved OVA (5 μg/ml) alone (triangles), with CNBr-cleaved OVA plus β_2-microglobulin (10 μg/ml) (circles), or with CNBr-cleaved OVA, β_2-microglobulin, plus 5% mouse serum (squares), washed, and treated with paraformaldehyde. The indicated number of fixed EL4 APCs was then incubated with 10^5 OVA- and K^b-specific T-cell hybrid RF33.70 cells in RPMI medium containing 10% fetal calf serum. After an 18-h incubation at 37°C, 100 μl of culture supernatant was removed from duplicate microcultures and assayed for interleukin 2 content. Data are expressed as the mean incorporation of [^{3}H]thymidine (cpm) into HT-2 cells. (*B*) Similar to *A* except the synthetic peptide OVA-(257–264) (0.4 ng/ml) was used instead of CNBr-cleaved OVA. Data in *A* and *B* are from the same experiment.

nous peptide with cell surface MHC class I molecules (1–4). Fig. 1*A* illustrates this effect for CNBr-cleaved OVA presentation. The presence of free human β_2-microglobulin during incubation of EL4 APCs with CNBr-cleaved OVA peptides augments their presentation to the OVA- and K^b-specific hybridoma RF33.70. In the same experiment, the addition of 5% (vol/vol) mouse serum during pulsing resulted in a marked increase in presentation compared to cells exposed to CNBr-cleaved OVA and β_2-microglobulin alone (Fig. 1*A*). This effect is most pronounced at limiting antigen concentration, which is the condition utilized in subsequent experiments. It is unlikely that free β_2-microglobulin present in mouse serum was responsible for the observed increase, as additional exogenous β_2-microglobulin does not result in enhanced presentation in this system (L.D.F., unpublished data).

The peptides that bind optimally to MHC class I molecules are 8–10 amino acids long (15–18). For OVA, the peptide OVA-(257–264) is likely to correspond to the naturally processed peptide (15). It is >1000-fold more potent than heterologous CNBr-cleaved OVA preparations or the synthetic peptide OVA-(258–276) (Fig. 1; K.L.R., unpublished results). We compared the effects of serum and β_2-microglobulin on the presentation of OVA-(257–264) versus CNBr-cleaved OVA. Similar to our observations with CNBr-cleaved OVA, free β_2-microglobulin augmented presentation of OVA-(257–264) (Fig. 1*B*). However, the addition of mouse serum during peptide pulsing reduced considerably OVA-(257–264) presentation (Fig. 1*B*). The reduction in presentation cannot be attributed to a general inhibitory effect of serum on the EL4 APCs during APC pulsing, as no inhibition is observed in the reciprocal experiment with serum and CNBr-cleaved OVA (Fig. 1*A*).

Serum Proteases Generate a Low Molecular Mass Antigenic Peptide from CNBr-Cleaved OVA. Serum inactivation of antigenic peptides has been observed and attributed to proteolysis (19). Similarly, the serum enhancement of CNBr-cleaved OVA presentation we observed may be a consequence of proteolysis. In this case, serum proteases may cleave long peptides in CNBr-cleaved OVA preparations containing residues 257–264 into smaller more-potent antigenic peptides. To test this hypothesis, we preincubated CNBr-cleaved OVA in a cell-free system with or without 5% mouse serum for 3 h at 37°C and assayed the resulting low molecular mass (<3 kDa) fraction for functional activity (Fig. 2*A*). The low molecular mass fractions from serum-exposed CNBr-cleaved OVA preparations were markedly more antigenic than unexposed CNBr-cleaved OVA preparations. In the same experiment, CNBr-cleaved OVA preincubation with serum at 4°C did not enhance antigenicity of the low molecular mass filtrate (Fig. 2*B*). This demonstrates the temperature dependence of this effect. Furthermore, it is unlikely that a serum component with a molecular mass <3 kDa is responsible for the enhanced antigen presentation, as it would be present in the filtrate prepared at 4°C. Together, these results indicate that incubation of CNBr-cleaved OVA with serum results in the generation of a low molecular mass

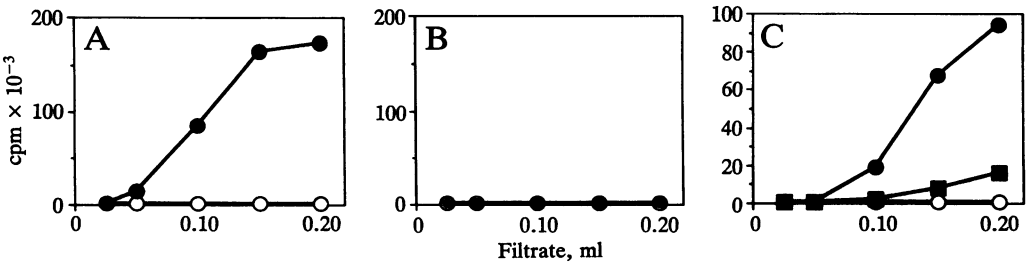

FIG. 2. Preincubation of CNBr-cleaved OVA with serum augments presentation of low molecular mass fraction of CNBr-cleaved OVA. (*A*) CNBr-cleaved OVA (60 μg/ml) was preincubated at 37°C in complete serum-free Opti-MEM (open circles) or in Opti-MEM plus 5% mouse serum (solid circles) for 3 h. Subsequently, 2 ml of each sample was centrifuged through a Centricon-3 filter (Amicon) and the indicated volume of each filtrate was incubated with 10^6 EL4 cells per ml plus β_2-microglobulin (10 μg/ml) in serum-free complete Opti-MEM for 3 h at 37°C in a total volume of 1 ml. EL4 cells were then washed, treated with paraformaldehyde, and added to cultures with RF33.70 cells. (*B*) Similar to *A* except preincubation was at 4°C. (*C*) Similar to *A* except an additional sample consisting of CNBr-cleaved OVA (60 μg/ml) and 5% mouse serum was preincubated for 3 h at 37°C in the presence of leupeptin (squares; 1 mg/ml); and leupeptin (200 μg/ml) was added directly to cultures of RF33.70 and EL4 APCs that had been preincubated with CNBr-cleaved OVA plus 5% mouse serum as in *A*. Cultures were prepared, handled, and assayed as described in Fig. 1. Data in *A* and *B* are from the same experiment.

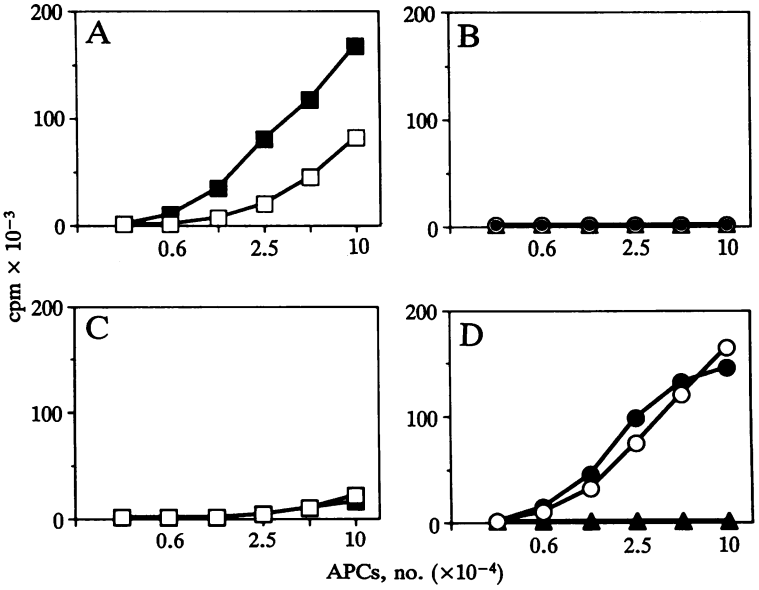

FIG. 3. Leupeptin inhibits serum augmentation of CNBr-cleaved OVA presentation but not serum inhibition of OVA-(257–264) presentation. EL4 cells were incubated in complete Opti-MEM (serum free) for 3 h at 37°C with the following components. (*A*) CNBr-cleaved OVA (3 μg/ml), β_2-microglobulin (10 μg/ml), and 5% mouse serum (solid squares) or with CNBr-cleaved OVA, β_2-microglobulin, mouse serum, and leupeptin (300 μg/ml) (open squares). (*B*) CNBr-cleaved OVA (3 μg/ml) alone (triangles), CNBr-cleaved OVA and β_2-microglobulin (10 μg/ml) (solid circles), or CNBr-cleaved OVA, β_2-microglobulin, and leupeptin (300 μg/ml) (open circles). EL4 cells were then washed and treated with paraformaldehyde, and the indicated number of treated EL4 APCs was added to culture with RF33.70 cells. Conditions in *C* and *D* are similar to *A* and *B*, respectively, except the synthetic peptide OVA-(257–264) (0.4 ng/ml) was used instead of CNBr-cleaved OVA. Cultures were prepared, handled, and assayed as described in Fig. 1. All data are from the same experiment.

antigenic peptide through a temperature-dependent mechanism.

To further define the mechanism of serum enhancement of CNBr-cleaved OVA presentation, we examined the effect of the competitive protease inhibitor leupeptin on serum enhancement. The presence of leupeptin during serum preincubation of CNBr-cleaved OVA resulted in greatly reduced antigenicity of CNBr-cleaved OVA filtrates (Fig. 2*C*). When EL4 cells are exposed to CNBr-cleaved OVA or OVA-(257–264), leupeptin does not inhibit subsequent antigen presentation (Fig. 2). Thus the observed leupeptin inhibition occurs during the preincubation of CNBr-cleaved OVA with serum, before APC pulsing. Inhibition by leupeptin implicates proteolysis as a mechanism of serum enhancement of CNBr-cleaved OVA presentation.

Serum Contains at Least Two Proteolytic Activities That Differentially Affect Peptide Antigenicity. To further assess the role of serum proteases on peptide antigenicity, we examined the effect of various protease inhibitors on the presentation of CNBr-cleaved OVA and OVA-(257–264). EL4 APCs were pulsed with limiting concentrations of peptide plus β_2-microglobulin with and without serum, in the presence or absence of protease inhibitors. When leupeptin was added under these conditions, serum enhancement of CNBr-cleaved OVA presentation was significantly inhibited (Fig. 3*A*). In the same experiment, leupeptin had no effect on inhibition by serum of OVA-(257–264) presentation (Fig. 3*C*). Interestingly, we found that the protease inhibitor bestatin, an exopeptidase inhibitor that differs from leupeptin in peptidase specificity, gives a reciprocal pattern of effect. Under identical conditions, bestatin had no effect on serum enhancement of CNBr-cleaved OVA presentation (Fig. 4*A*) but prevented serum inactivation of OVA-(257–264) (Fig. 4*C*). In the latter case, nonspecific stimulation by bestatin is unlikely, as CNBr-cleaved OVA presentation is unaffected under identical conditions (Fig. 4). Thus these results demonstrate that serum contains at least two protease activities: a leupeptin-sensitive endopeptidase that enhances the antigenicity of CNBr-cleaved OVA and a bestatin-sensitive exopeptidase that decreases the antigenicity of OVA-(257–264).

DISCUSSION

Any effect of serum on the antigenicity of peptides is potentially relevant to their availability *in vivo* and for their use as immunogens. Our studies suggest that serum contains pro-

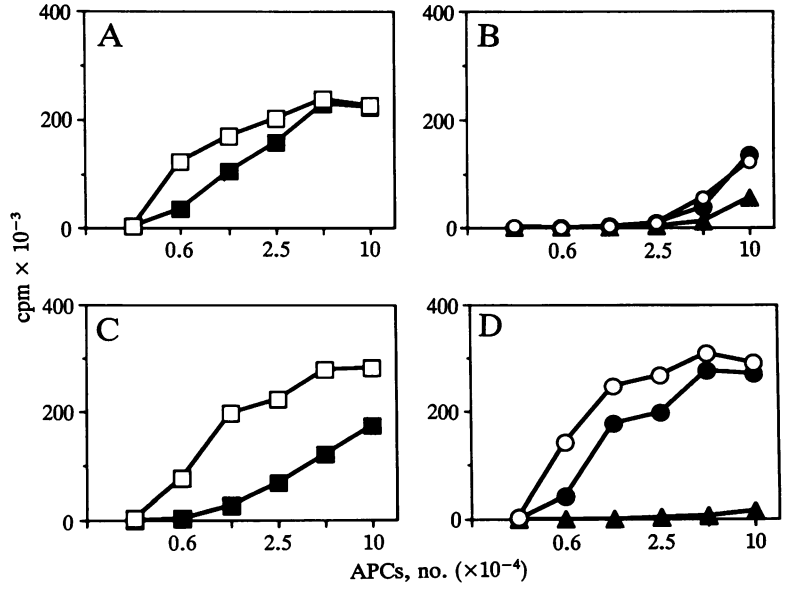

FIG. 4. Bestatin prevents serum inhibition of OVA-(257–264) presentation but not serum augmentation of CNBr-cleaved OVA presentation. EL4 cells were incubated in complete Opti-MEM (serum free) for 3 h at 37°C with the following components. (*A*) CNBr-cleaved OVA (3 μg/ml), β_2-microglobulin (10 μg/ml), and 5% mouse serum (solid squares) or CNBr-cleaved OVA, β_2-microglobulin, mouse serum, and bestatin (250 μg/ml) (open squares). (*B*) CNBr-cleaved OVA (3 μg/ml) alone (triangles), CNBr-cleaved OVA and β_2-microglobulin (10 μg/ml) (solid circles), or CNBr-cleaved OVA, β_2-microglobulin, and bestatin (250 μg/ml) (open circles). EL4 cells were then washed and treated with paraformaldehyde, and the indicated number of treated EL4 APCs was added to culture with RF33.70 cells. Conditions in *C* and *D* are similar to *A* and *B*, respectively, except the synthetic peptide OVA-(257–264) (0.4 ng/ml) was used instead of CNBr-cleaved OVA. Cultures were prepared, handled, and assayed as described in Fig. 1. All data are from the same experiment.

teases that can affect the antigenicity of peptides in distinct ways.

By using a functional assay, we demonstrate that a serum component enhances the presentation of CNBr-cleaved OVA. This serum activity is present in a cell-free system and has three features that implicate proteolysis: (*i*) It is temperature dependent. (*ii*) It results in increased antigenicity in a low molecular mass (<3 kDa) peptide fraction. (*iii*) It is inhibited by the protease inhibitor leupeptin. With the same functional assay, we observed decreased antigenicity of OVA-(257–264) in the presence of serum. This inactivation was unaffected by the presence of leupeptin but was blocked by bestatin.

Our data indicate that CNBr-cleaved OVA preparations contain the naturally presented OVA-(257–264) sequence within a longer peptide. One interpretation of these results is that a leupeptin-sensitive protease in serum cleaves an OVA-(257–264)-containing peptide into a smaller more-potent antigen, resulting in enhanced functional presentation of CNBr-cleaved OVA. Conversely, proteolysis of OVA-(257–264) results in a less-antigenic peptide. That decreased presentation of OVA-(257–264) in the presence of serum is inhibited by bestatin but not by leupeptin suggests that serum contains proteases with at least two substrate specificities. Given these results, it is not surprising that prolonged incubation of CNBr-cleaved OVA with serum results in a loss of antigenic activity (L.D.F., unpublished data). These results imply that once peptides are bound to MHC class I, they are resistant to further proteolysis.

Leupeptin and bestatin inhibit different classes of proteases. Leupeptin is an endopeptidase inhibitor and has been shown to inhibit serine and thiol proteases such as trypsin, papain, plasmin, and cathepsin B (20–22). Bestatin is an inhibitor of aminopeptidases and other exopeptidases including leucine aminopeptidase, aminopeptidase B, and tripeptide aminopeptidase (21, 22). It is likely that additional proteases present in serum can affect peptide antigenicity. In our system, we found no effect on either CNBr-cleaved OVA or OVA-(257–264) presentation by the protease inhibitors chymostatin, pepstatin, or captopril (unpublished data) in the presence of serum. Our functional studies are limited to OVA plus K^b presentation. Presentation of other antigens would potentially be affected by proteases with different substrate specificities. In this regard, Widmann *et al.* (19) have demonstrated that several antigenic peptides can be inactivated by serum proteases. While our manuscript was in preparation, Sherman *et al.* (23) reported that a serum protease enhances the presentation of an influenza nucleoprotein peptide. The enzyme responsible for this effect was identified as serum angiotensin-converting enzyme. Similarly, Kozlowski *et al.* (24) demonstrated that serum angiotensin-converting enzyme enhanced presentation of the human immunodeficiency virus 1 gp160-derived peptide p18.

Many studies have analyzed the sequence and length requirements of peptide binding to class I molecules on cultured cells. Our findings and those of Sherman *et al.* (23) and Kozlowski *et al.* (24) suggest that the potential effects of serum proteases in such assays need to be considered.

Successful use of peptides to prime CTL responses *in vivo* will require peptide persistence sufficient to favor peptide–MHC class I binding. Persistence of peptide antigens *in vivo* will be limited by clearance and degradation. The observations reported here demonstrate that the antigenicity of peptides can be increased or decreased by serum proteases. They raise the possibility that the ideal immunogen may be a precursor or modified version of the naturally processed 8- to 10-amino acid peptide antigen. The effect of serum proteolysis on antigenicity should be considered as *in vivo* CTL priming protocols are developed.

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