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ESTIMATION OF CELL SURFACE ASSOCIATED PROTEASE ACTIVITY AND ITS APPLICATION TO LYMPHOCYTES

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A new method has been developed to estimate proteolytic activity available at the cell surface. Radioiodinated protein substrates are covalently linked to modified polystyrene-divinylbenzene beads with various diameters. These beads are presented to viable cells. Secreted enzyme activity is estimated when no contact occurs between beads and cells. Surface associated proteolytic activity is estimated by the increased rate of iodinated peptide release due to a contact between beads and cells.

This method was applied to various lymphocyte preparations. In the absence of serum, mouse spleen lymphocytes produce three- to fourfold higher proteolytic activity than lymph node cells. This activity is completely inhibited by serum diluted 1:10. Since the proteolysis is so marked in the case of spleen cells, one must conclude that lymphocytes removed from the serum and treated in buffered mediums at 37°C have enzymatically altered surface properties.

Cell surface associated enzyme activity was measured using rat lymph node lymphocytes with less than 0.1% contamination by granulocytes. This predominantly thymus derived, T cell population had 30% increase in proteolysis due to contact between cells and solid-phase localized substrate of casein. The released enzymatic activity was inhibited by diisopropylfluorophosphate, but its effect on the surface associated enzyme activity remains questionable since it perturbs several membrane functions.

INTRODUCTION

Proteolytic enzymes restricted to the two-dimensional topography of a cell membrane are of interest because their active sites may only be available to the external environment of the cell. These enzymes could therefore alter the surrounding of the cell without affecting its membrane, inflict damage on another cell (as in the case of cell mediated cytotoxicity), or be involved in selective shedding of specific surface components. Because of these wide biological implications, a direct method to estimate the

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amount of enzyme activity and its mode of action on viable cell surfaces is of considerable importance.

We now report an approach whereby the substrate, a protein previously labeled with ^{125}I protein, is covalently linked to modified polystyrene divinylbenzene particles. The viable cells are incubated with or without contact with the substrate complex. The rate of iodinated peptide release is proportional to the enzyme activity, and the increase in rate, due to contact, gives an estimation of the amount of enzyme activity available at the viable cell surface. This method is relatively simple and requires only 10^5-10^6 cells per determination. It is very sensitive and allows flexibility in choice of substrates. After measurements, the cells can be recovered with insignificant loss in viability. The method, however, must be used with considerable caution if cells such as granulocytes, capable of secreting large amounts of proteases, are present.

MATERIALS AND METHODS

Human erythrocyte membrane was prepared as previously described (1). DA rat lymph node cells were kindly provided by Dr. Haim Ginsburg. Axillary, brachial, inguinal, and mesenteric nodes were removed and placed in Hank's medium at room temperature. Single cell suspension was prepared under sterile conditions by teasing the nodes with forceps and Dispo-pipettes. The cells were washed by repeated sedimentation. These preparations were 99% viable as estimated by trypan blue exclusion and contained less than 0.1% granulocytes. Over 80% of these cells were capable of transforming to blast cells by pokeweed mitogen and lysed syngeneic fibroblasts grown in a monolayer. Lymph node cells from Balb/C mice were prepaıed similarly. Spleen lymphocytes were kindly prepared by Dr. R. Cone. The removed spleen was gently teased in cold Medium 199 containing 1% antibiotic-antimycotic mixture and 2% inactivated fetal calf serum. The single cell suspension was layered on Ficoll-Urovision, density 1.077, and centrifuged for 15 min at 1,500 rpm in MSE-Mistral centrifuge. The cell layer at interface was recovered and diluted twofold with cold Medium 199. The cells were washed free of Ficoll by repeated centrifugation. Viability was 95–99%. These preparations usually contained 2–8% granulocytes.

Various protein preparations were labeled with ^{125}I using the lactoperoxidase method as previously described (2). Neutral-protease free casein, water soluble carbodiimide coupling reagent – 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluene sulfonate – and the modified polystyrene divinylbenzene latex beads with uniform size were obtained from Bio-Bifunctionals, Los Angeles, California. These beads have covalently linked amino groups and are available in various uniform sizes with average diameters of 17, 26 and 45 μ. The proteins are covalently linked to the beads by the formation of an amide bond using carbodiimide as previously described (1). Adsorbed proteins are washed off by repeated centrifugation in 5 molar guanidine-HCl. Washing is continued until the amount of radioactive material released from the beads is less than 0.2% and does not change by additional washings. Nonspecific adsorption of radioactive proteins can be minimized if the beads are also treated with 1% neutral-protease free serum albumin after repeated washings. The substrate-bead complexes are relatively stable if stored with 1% NaN_3 at 0°C for less than 10 days. Prior to each experiment they should be washed with the desirable buffers by repeated centrifugation. In general we found that a surface density of 1–3 radioiodinated protein molecules, approximate molecular weight of 50,000 per 100 square Å was necessary to obtain reliable results.

All incubations were carried out under sterile conditions at 37°C in the presence of penicillin and streptomycin (Grand Island Biochemical Co., New York). Controls used equal volumes of 1% human serum albumin (HSA), electrophoretically pure (Behringwerke AG, Marburg-Lahn, West Germany).

Erythrocyte membrane preparations were diluted to 0.6 mg protein per ml with phosphate buffered saline (PBS), pH 7.9, containing 0.01% NP-40 detergent. 100 μl of this membrane preparation was incubated with 50 μl of substrate-bead suspension containing 4×10^4 cpm ^{125}I on casein covalently linked to approximately 2×10^4 modified beads with 26 μ average diameters. Total radioactivity was measured in a Packard 5260 Autogamma Scintillation Spectrometer. Incubation was stopped by adding 1.0 ml of cold 1% HSA and by centrifuging the beads down at 1,000 rpm for 3 min in the cold. 1.0 ml of the supernatant was removed, and the percent of total radioactivity delocalized from the beads was determined.

To estimate total proteolytic activity, 4×10^6 mouse spleen or lymph node lymphocytes were suspended in 100 μl of Hank's or Delbucco's medium. The cells were mixed with 2×10^4 cpm ^{125}I-casein covalently linked to 26 μ diameter beads and suspended in 50 μl of PBS. Supernatants obtained from an equal number of cells preincubated for 1, 2, and 3 hr under identical conditions were further incubated with the substrate for 1, 2, and 3 hr, respectively. Identical supernatant samples were also treated with 1 μl diisopropylfluorophosphate (DFP) (Fluka AG, Buchs S.G., Switzerland) and tested for enzyme activity. Diisopropylphosphate was prepared by incubating DFP in PBS at 40°C for 60 min. After this incubation no more trypsin inhibition was obtained, indicating a complete hydrolysis of DFP to diisopropylphosphate.

Contact between cells and beads was established when they cosedimented during incubation. 100 μl of 1% HSA in PBS, pH 7.9, with or without DFP was used as control. All incubations were done in triplicate. Proteolytic activity is expressed as the percent of radioactivity released from the beads as a result of incubation. The effect of serum was studied using syngeneic mouse or rat serum at various dilutions.

RESULTS

We have chosen the modified polystyrene divinylbenzene latex beads as supporting medium because of their availability in various uniform sizes and their low porosity. Of the various sizes we found the 26 μ particle size the most suitable. Smaller particles may get phagocytized and larger particles, with 45 μ diameter, settle down too fast, which makes pipetting less accurate. We have also tried various radioiodinated protein substrates, such as horse heart cytochrome-c, hemoglobin, human and mouse immunoglobulins, and casein. Cytochrome-c and hemoglobin were found to be difficult to digest. The sensitivity of immunoglobulins varied with the intactness of their domains, indicating that additional conditions, such as the presence or absence of Fc receptors, were complicating our observations (3). In all the experiments presented here, ^{125}I-casein was used as substrate. The released radioactivity was associated with polypeptides, smaller than the original protein used for coupling as judged by sodium dodecylsulfate polyacrylamide gel electrophoresis. Since the molecular weights varied we infer a random digestion.

In Fig. 1, the rate of ^{125}I release by the erythrocyte membrane preparation is illustrated. As previously reported (1), this membrane contains restricted proteolytic activity. The rate of ^{125}I release was linear until approximately 20–25% of the radioactivity was removed from the beads. The rate was also proportional to the amount of

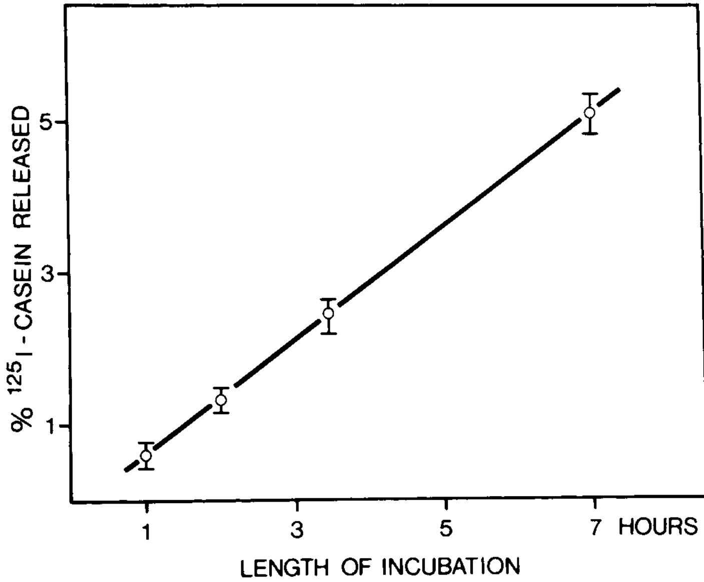

Fig. 1. The rate of proteolytic digestion of ^{125}I-casein by erythrocyte membrane preparation. Values for the controls without membranes were subtracted. Vertical bars represent standard deviations from three determinations.

membrane preparation added. Under similar conditions 0.5–0.7% ^{125}I-label was released by 40 ng trypsin per hour.

When viable cells and substrate-bead complexes are incubated together, the rate of ^{125}I-peptide release indicates the total proteolytic activity. This activity is a summation of the released enzymes and of possible cell surface associated enzymes (Fig. 2). This method

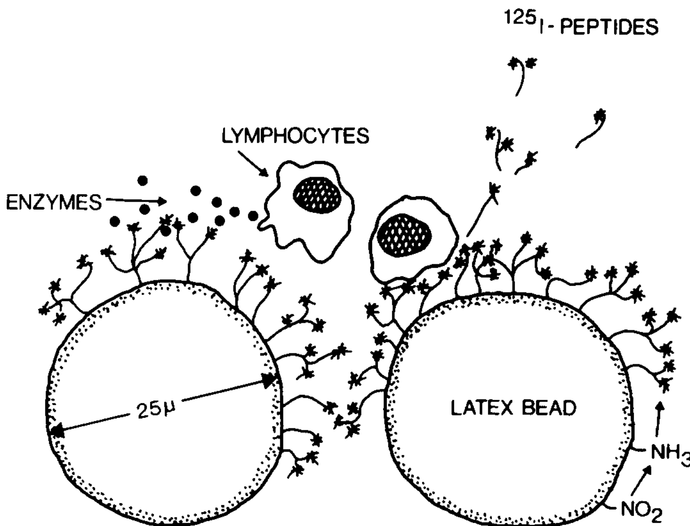

Fig. 2. Graphic illustration of the beads' application to estimate total proteolytic activity associated with viable lymphocytes.

was applied to mouse spleen and lymph node lymphocytes in the presence and absence of syngeneic serum. The results (Fig. 3) illustrate the substantial proteolysis produced by the spleen cells. If, however, the bone-marrow-derived, B cell type lymphocytes were further purified from the spleen cell population, the difference between lymph node cells and spleen cells would diminish. The most significant contribution was from granulocytes (Tökés and Trinchieri, unpublished observations). Total proteolytic activity by spleen cells was 95% inhibited by serum diluted to 1:10. This extent of inhibition is partly due to substrate competition. Serum albumin, however, was less efficient in inhibition, which suggests the additional effect of serum protease inhibitors. Diluted serum alone also exhibited a residual activity with the radioiodinated casein.

Difficulties were encountered in trying to estimate cell surface associated activities on spleen cells because of the high amount of proteolytic activities released by them. However, results from rat lymph node cells are summarized in Fig. 4. Incubations were carried out in the absence of serum for 3 hr. During this time the viability did not decrease more than 5%. Complications may arise due to released enzymes when lysed cells are present. During the first hour of incubation, only a slight increase was observed in the rate of ^{125}I release when the cells were coincubated with the beads. During this time, contact is established. This enhancement gradually increased and after 2 hr 30% more label was delocalized from the beads.

The same results were observed whether or not the incubation mixture was gently rocked to assure an even distribution of released enzyme supernatant. This indicates that

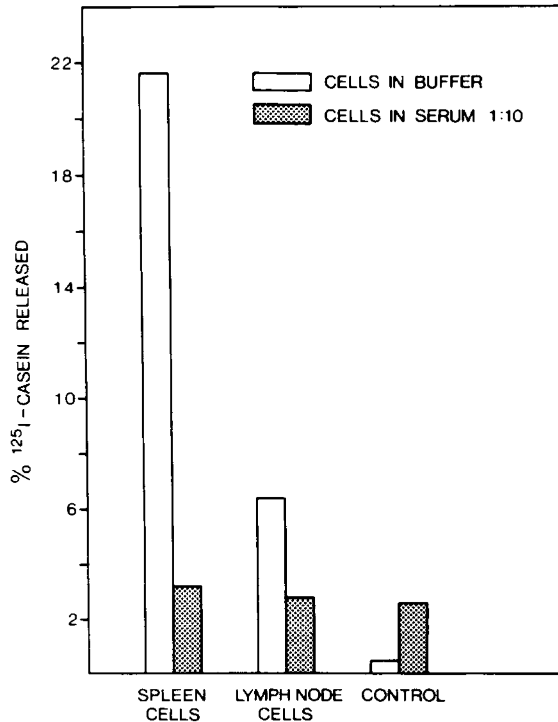

Fig. 3. Total proteolytic activity of mouse spleen and lymph node lymphocytes in the presence or absence of syngeneic serum. All incubations were carried out for 2 hr at 37°C.

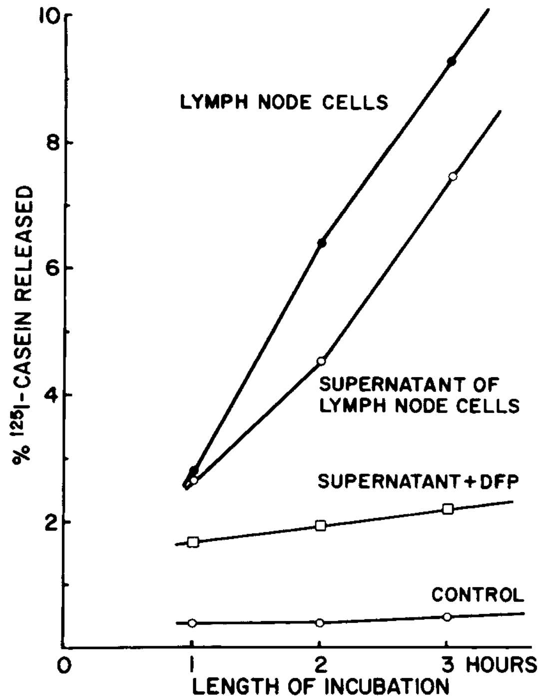

Fig. 4. Proteolytic activity of rat lymph node lymphocytes and the effect of diisopropylfluorophosphate on the activity of lymphocyte supernatant. All incubations were carried out at 37°C in Delbucco's medium without any serum.

the enhancement is due to contact and not due to an increased "local" concentration of enzymes in the vicinity of cell-bead complexes.

DFP inhibited most of the enzyme activity released into the supernatant. However, this inhibitor also lysed a significant portion of the cells and usually resulted in the release of cytoplasmic enzymes. Diisopropylphosphate, an inactive breakdown product of the inhibitor, was absorbed to the cell's lipid membrane, as measured by the ^{3}H-labeled compound. Therefore, this inhibitor's function with the cell surface associated enzymes could not be accurately determined.

DISCUSSION

We have presented an approach to estimate cell surface associated proteolytic activity. This method was developed to bypass the trichloroacetic acid precipitation step commonly used in most assays, and to offer flexibility in the choice of substrates, a choice not available in the fibrinolysis assays. The method also eliminates possible phagocytic degradation because the substrate is covalently linked to a solid-phase support. The sensitivity allows one to measure 10–20 ng of trypsin-equivalent activity during incubation for 2–3 hr.

Cationic protein substrates linked to the beads, such as cytochrome-c and histones, although poorly iodinated, can form a rosette type of cell aggregation. Aggregated immunoglobulins can selectively recruit lymphocytes to the beads by the F_c receptor mechanism. These additional features will allow one to study surface proteases under a variety of

conditions. The approach is also used currently in our laboratories for testing various biological materials for specific inhibitory activity of proteases available on the cell surfaces and for testing the effect of various mitogens on lymphocytes.

When studying lymphocytes we have found a consistent difference between B and T cell populations. This, however, always varied with the source of cell preparation and usually indicated minor populations of contaminating cell types, such as granulocytes, whose total proteolytic activity is several-fold higher than that of small lymphocytes. For this reason, the method has to be employed with caution.

Significant increase in proteolytic activity was observed when cells were brought in contact with the beads (Fig. 4). The onset of the increase varied with experimental conditions. If cells and beads were allowed to settle slowly under 1 G conditions, the difference occurred after the first hour of incubation. This sedimentation could be increased if the mixture was cosedimented by centrifugation at 500 rpm for 2 min in a clinical table-top centrifuge. Usually a 30–40% increase was observed with lymph node cells by the second hour of incubation. This amount of activity corresponds to about 0.18 μg "trypsin equivalent" activity per 4×10^6 cells, although such correlations are not to be used for true comparison, since the behavior of an enzyme restricted to the plasma membrane's topography cannot be compared with enzymes in solution.

The effect of DFP on the cell surface associated enzyme activity is difficult to interpret. Whereas some inhibition was observed after two hours of incubation, shorter incubations resulted in no change or a moderate increase in the overall enzyme activity. This is most likely due to secondary effects of this inhibitor, since the addition of the breakdown product, diisopropylphosphate, resulted in similar observations. The neutral protease activity released by lymphocytes into the supernatant is predominantly derived from enzyme(s) containing serine in their active sites, since DFP inhibits irreversibly. Whether these released enzymes become associated with the cell membrane, is now under investigation. It is also difficult at this time to determine the possible biological roles for cell surface proteases since they may be completely inhibited in the presence of serum. Even the origin of this enzyme activity is not certain. Enzyme-inhibitor complexes may adsorb to cells in the plasma, and repeated washing could selectively remove more inhibitors than enzymes.

The approach we have described here is also applicable to cells grown in tissue culture. Currently we are using a similar method to study normal and viral transformed fibroblasts and their surface proteases.

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