Flow Cytometry to Identify Cell Types to Which Enzymes Bind

**EFFECT OF LACTIC DEHYDROGENASE VIRUS ON ENZYME BINDING**

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Flow cytometry was used to measure the binding of enzymes (i.e. lactate dehydrogenases 1 and 5, malate dehydrogenase, and asparaginase) to cells. Of the four enzymes studied, asparaginase showed the greatest binding. Single color analysis revealed that asparaginase bound best to preparations enriched in macrophages, and dual color analysis showed that the binding was to macrophages. Studies on continuous cell lines revealed that asparaginase bound to one mouse macrophage line, but not to another or to murine fibroblasts. Inoculation of mice with lactic dehydrogenase was to macrophages. Studies on continuous cell lines revealed that asparaginase bound to one mouse macrophage line, but not to another or to murine fibroblasts. Inoculation of mice with lactic dehydrogenase virus (LDV), which infects macrophages, showed the in vivo clearance of asparaginase from the circulation and the in vitro binding of asparaginase to peritoneal macrophages. It is concluded that flow cytometry can be used to study the binding of enzymes to cells, to identify the cell type to which the enzyme binds, and to measure changes in the capacity of cells to bind enzymes.

Alterations of enzyme levels in the blood are important for the diagnosis of certain diseases (Zimmerman and Henry, 1984). Enzyme elevation is generally attributed to release of enzymes from damaged tissue; but, in fact, the enzyme level in the blood represents the balance between enzyme influx and enzyme clearance (Hayashi et al., 1988; Notkins, 1965). Some enzymes are thought to be released by the reticuloendothelial system (RES) (Bijsterbosch et al., 1981, 1982; DeJong et al., 1981; Sinke et al., 1979). Factors which affect RES (Hayashi et al., 1988; Mahy et al., 1967; Notkins, 1965) function have been shown to influence enzyme clearance. For example, mice inoculated with lactic dehydrogenase virus (LDV), which infects macrophages, show a lifelong 5–10-fold increase in the level of several plasma enzymes and a marked decrease in enzyme clearance (Hayashi et al., 1988; Mahy et al., 1964; Notkins, 1965; Notkins and Scheele, 1964). Agents which inhibit macrophage function, such as silica, also can produce long-term impairment of enzyme clearance and increase plasma enzyme levels (Hayashi et al., 1988).

Current methods for studying enzyme clearance in animals involve injecting a known amount of enzyme intraperitoneally or intravenously and monitoring, over time, the disappearance of the enzyme from the circulation. Currently, there is no good in vitro correlate, and information on the specific cell types involved is limited. This study, which utilizes flow cytometry, was undertaken: 1) to analyze the first step in enzyme clearance, the binding of enzymes to cells; 2) to identify the specific cell type(s) involved; and 3) to examine factors which affect the binding of enzymes to cells.

**MATERIALS AND METHODS**

**Animals**—Seven- to nine-week-old male BALB/cAnN mice were obtained from the Animal Production Section of the National Institutes of Health (Bethesda, MD). They were kept in plastic cages with wood chip bedding and given pellets and water *ad libitum*.

**Infected Proteins—Escherichia coli** asparaginase, porcine muscle lactate dehydrogenase 5, porcine heart lactate dehydrogenase 1, and malate dehydrogenase were obtained from Sigma. Enzymes were dissolved in 0.1 M NaHCO$_3$, pH 0.9, to 1 mg/ml. $N$-Hydroxysuccinimidobiotin (Pierce Chemical Co.) was dissolved in dimethyl sulfoxide (Sigma) to 1 mg/ml and was added to the enzyme solution (125 μg of $N$-hydroxysuccinimidobiotin/ml of enzyme solution). After 3–4 h of incubation at room temperature, the mixture was dialyzed against phosphate-buffered saline (Quality Biological, Inc., Gaithersburg, MD) at 4 °C for 2–3 days. Dialyzed biotinylated proteins were concentrated by ultrafiltration with a Centricon 30 microconcentrator (Amicon Corp.) and centrifuged at 10,000 × g for 5 min prior to use.

Confirmation that lactate dehydrogenases 1 and 5 were biotinylated was accomplished by dot-blot analysis using peroxidase-labeled avidin.

**Cell Culture**—Mice were injected intraperitoneally with thioglycolate medium (Difco). Four days post-injection, mice were killed by CO$_2$ asphyxiation, and the peritoneal cavities were washed with Hanks’ balanced saline solution (Biofluids, Rockville, MD). The retrieved fluid was collected and centrifuged at 1000 rpm for 10 min. Sedimented cells were washed three times with Hanks’ balanced saline solution and resuspended in RPMI 1640 medium (Biofluids) including 10% fetal calf serum (GIBCO), and the cell suspension was plated in 60 × 15-mm tissue culture dishes (Costar, Cambridge, MA). After overnight incubation, adherent cells were collected by scraping and used for further experiments. Splenic adherent cells were prepared by macerating mouse spleen in Hanks’ balanced saline solution, lysing the red cells with Tris-buffered NH$_4$Cl solution, and incubating the resulting cell suspension in 10% fetal calf serum/RPMI 1640 overnight, as described above, to separate adherent and nonadherent cells. Peritoneal and splenic adherent cells were tested for phagocytic activity by inoculation with latex particles (Sigma), followed by microscopic observation. More than 90% of the cells demonstrated particle uptake.

Mouse macrophage cell lines IC21 and RAW 264.7 as well as a mouse embryo cell line (BALB/3T3) were obtained from American Type Culture Collection (Bethesda, MD) and cultured as recommended by the supplier.

**LDV Infection—Plasma**—From an LDV-infected mouse which had a titer of $10^9$ ID$_{50}$/ml, as determined by the method reported previously (Notkins and Shochat, 1965), was diluted with RPMI 1640 medium containing 8% fetal calf serum. For *in vivo* infection, mice were injected intraperitoneally with 1.0 ml of thioglycolate and 0.1 ml of dilute plasma containing 10$^9$ ID$_{50}$/ml LDV. Four days later, peritoneal cells were collected, and adherent cells were cultured as described above. For *in vitro* infection, thioglycolate-stimulated peritoneal adherent cells were collected from uninfected mice and cul-

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The abbreviations used are: RES, reticuloendothelial system; LDV, lactic dehydrogenase virus; FITC, fluorescein isothiocyanate.
tured. Cells were infected with LDV at 10^3 ID50/cell, and 12 h later were used in the enzyme binding experiments.

**In Vivo Asparaginase Clearance**—Mice were infected intraperitoneally with 10^3 ID50 of LDV. Seven days later, infected and uninfected mice were injected intraperitoneally with 100 units of asparaginase in 0.2 ml of phosphate-buffered saline. Immediately after the asparaginase injection, mice were bled by the retro-orbital bleeding technique. Blood samples were taken 2, 4, 6, and 10 h later. Asparaginase activity in plasma was measured (Wriston, 1970) and clearance was calculated by methods described elsewhere (Notkins and Scheele, 1964).

**Cell Analyses**—Cells (2 x 10^6) were suspended in 0.1 ml of fluorescence-activated cell sorter buffer (0.1% bovine serum albumin and 0.1% sodium azide in phosphate-buffered saline and incubated at 4 °C with or without 50 μl of biotinylated enzyme solutions (500-1500 μg/ml) for 25 min. The cells were then washed twice and incubated with 100 μl of fluorescein isothiocyanate (FITC)-avidin (Sigma) (5 μg/ml) in fluorescence-activated cell sorter buffer at 4 °C for 25 min. After washing, cells were fixed with 2% paraformaldehyde. Analysis was performed using a FACS 440 flow cytometer (Becton, Dickinson & Co., Mountainview, CA), and fluorescence intensity was expressed by a logarithmic scale.

For dual color staining, 2 x 10^6 cells were first incubated with 1% normal mouse serum to block Fc receptors (Inghirami et al., 1988) and then with a mixture of rat anti-mouse macrophage monoclonal antibody (clone M1/70.15, Sera Laboratories, Crawley, England) (50 μl of a 1:10 dilution) and biotinylated asparaginase (70 μg), followed by a mixture of FITC-avidin (1 μg) and phycoerythrin-conjugated goat anti-rat IgG (Pana Data System, Rockville, MD) (1 μg). Data are expressed using logarithmic scales for both colors. The excitation wavelength for both FITC and phycoerythrin was 488 nm, and the emission wavelengths were 525 and 575 nm, respectively.

**RESULTS**

**Fluorocytometric Analysis of Enzyme Binding to Peritoneal Macrophages**—Biotinylated lactate dehydrogenases 1 and 5, malate dehydrogenase, and asparaginase were incubated with peritoneal macrophages, and the binding of the enzymes to macrophages was analyzed by flow cytometry. As seen in Fig. 1, lactate dehydrogenase 1 showed no binding, lactate dehydrogenase 5 minimal binding, and malate dehydrogenase moderate binding, whereas asparaginase bound to over 80% of the cells. Asparaginase was used in all subsequent experiments.

**Binding of Asparaginase to Different Cell Types**—The binding of asparaginase to different cell types is illustrated in Fig. 2. Asparaginase bound extensively to both peritoneal and splenic macrophages. The enzyme also bound to one macrophage line (RAW 264.7), but not to another macrophage line (IC21). Nonadherent splenic cells, depleted of most macrophages, showed relatively little binding; and mouse fibroblasts (BALB/3T3) showed essentially no binding.

Proof that the asparaginase was binding to macrophages in the peritoneal exudate population comes from analysis by dual color flow cytometry. As seen in Fig. 3, a rat anti-mouse macrophage antibody labeled to give red fluorescence identified the macrophage population (Fig. 3C), and biotin-labeled asparaginase incubated with FITC-avidin identified the cell population to which the asparaginase bound (Fig. 3B). Dual fluorescence showed that the asparaginase bound to macrophages (Fig. 3D).

**Inhibition of In Vivo Clearance and In Vitro Binding of**
Asparaginase by LDV Infection—LDV infects macrophages and raises enzyme levels in the circulation by impairing enzyme clearance. This is illustrated in Fig. 4A, which shows that the rate of asparaginase clearance in LDV-infected animals is about one-half that in uninfected controls. To see if this impairment of enzyme clearance might be related to a decrease in enzyme binding, the binding of asparaginase to LDV-infected macrophages was determined. As seen in Fig. 4 (B and C), both in vivo and in vitro infected macrophages bound less asparaginase than did uninfected macrophages.

**DISCUSSION**

Flow cytometry has been used to measure the binding of a variety of molecules to cells. In this study, we use flow cytometry to measure the binding of several enzymes to cells. It is clear from the results in Fig. 1 that there is a substantial difference in the binding of different enzymes to the same cell type (peritoneal macrophages), ranging from no binding of lactate dehydrogenase 1 to high binding of asparaginase. Moreover, asparaginase binds to only certain cell types. These observations raise the possibility that there might be a specific receptor for asparaginase. In fact, a variety of receptors having different biological properties have been found on the surface of RES cells (Ashwell and Harford, 1982; Goldstein et al., 1979; Vlassara et al., 1986). However, competition experiments with a 10-fold excess of unlabeled asparaginase did not inhibit the binding of labeled asparaginase to macrophages. If a specific receptor for asparaginase exists on the surface of macrophages, it is present in great excess.

These experiments also show that the specific cell type (i.e., macrophages) in a mixed cell population to which asparaginase binds can be identified by use of appropriate cell-surface markers and dual immunofluorescence. This suggests that, in some cases, in vitro enzyme binding studies might serve as a correlate of in vivo enzyme clearance. LDV, which infects RES cells, dramatically impairs the clearance of circulating asparaginase and reduces the binding of asparaginase to both in vivo and in vitro infected macrophages. This decrease in enzyme binding (Fig. 4) is substantial, considering that less than 20% of macrophages become infected with LDV (Oldstone et al., 1974; Tong et al., 1977). Perhaps lymphokines produced by LDV-infected cells responsible for the down-regulation of enzyme binding. Moreover, macrophages are only one of the several cell types that make up the RES and may not necessarily be the major cell type responsible for the clearance of a particular enzyme. For example, cultured hepatic Kupffer cells have been shown to take up considerable lactate dehydrogenase 5 (Smit et al., 1987), which suggests that these cells, and not peritoneal macrophages, may be the principal cell type that removes lactate dehydrogenase 5 from the circulation.

Recent studies in mice showed that genetic factors determine whether an individual is a slow or fast enzyme clearer (Hayashi et al., 1988). Thus, the enzyme level in the blood following acute tissue damage may reflect, in part, differences among individuals in enzyme clearance. Whether sensitive flow cytometry enzyme binding studies will make it possible to distinguish slow from fast enzyme clearers and whether this technique can be applied to human peripheral blood leukocytes remain to be determined. Further studies on the binding of enzymes to subsets of RES cells should reveal new information about the properties of these cells and perhaps uncover specific dysfunctions in various disease states.

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