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# [36] Recombinant Influenza C Hemagglutinin-Esterase as a Probe for Sialic Acid 9-*O*-Acetylation

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All mammalian cells are covered with a dense and complex array of glycan chains. The sialic acids are a family of negatively charged 9-carbon sugars commonly found at terminal positions on these cell surface glycans. As such, they are strategically positioned to interact with the extracellular environment. In keeping with this, sialic acids show marked diversity in their presentation, being attached to underlying sugar chains in a variety of $\alpha$ ketosidic linkages and being modified in a number of ways.[1,2] This diversity affects sialic acid recognition by a variety of specific binding proteins of endogenous and exogenous origin.[1–4]

One of the most common of these sialic acid modifications is substitution of the hydroxyl group at the 9-carbon position with an *O*-acetyl ester. 9-*O*-acetylated sialic acids were once thought to be rare, species-specific peculiarities, but are now known to be widely expressed in many species and cell types. 9-*O*-Acetylated sialic acids have been identified in all mammals studied thus far, occurring particularly in some cell types of neuroectodermal and mesenchymal origin.[1,2] Much evidence indicates that 9-*O*-acetylated sialic acids are expressed in a developmentally regulated and cell-type/tissue-specific fashion. Immunohistochemistry using specific monoclonal antibodies indicates a temporal and spatial pattern of regulation of 9-*O*-acetylation in embryonic tissues. Moreover, for a given cell type, 9-*O*-acetylation appears to be distributed nonrandomly on specific cell surface glycoconjugates, as well as on certain types of sialic acid linkages.[5–8]

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It is known that 9-*O*-acetylation can affect the biological activity of various molecules, e.g., mediating recognition of cells by viruses,[9–11] modulating activation of the alternate pathway of complement,[12] and masking sialylated ligands for Siglecs such as CD22/Siglec-2 on B cells[13] and sialoadhesin/Siglec-1 on tissue macrophages.[14] Abrogation of 9-*O*-acetylation can also affect development in transgenic mice.[15] Earlier studies indicated that some human lymphocytes express *O*-acetylated sialic acids, whereas others did not,[16,17] and that T cells of patients with various malignancies were shown to acquire high levels of 9-*O*-acetylation.[18] In mice, sialic acids on $CD4^+$ T cells appear to be preferentially 9-*O*-acetylated,[19] suggesting a role in T-cell development and/or function.

The binding and cleavage properties of the *Influenza C* virions hemagglutinin-esterase (CHE) protein for 9-*O*-acetyl groups on sialic acids have been taken advantage of in various assays using whole virions.[20,21] Given the limitations of using whole virions for routine studies, we engineered a recombinant soluble molecule composed of the extracellular domain of CHE fused to the Fc region of human IgG (CHE-Fc).[22] Treatment of this chimeric molecule with diisopropyl fluorophosphate (DFP) selectively eliminates its esterase activity while preserving its binding property (CHE-FcD).[22] Thus, this probe serves as a bifunctional tool for selectively removing (with CHE-Fc) or specifically detecting (with CHE-FcD) 9-*O*-acetylated sialic acids.[5–7,14,19,22,23]

## Preparation of CHE-Fc Chimera

### *Cell Culture*

*Adherent Cells.* The CHE-Fc chimera is produced from human embryonic kidney 293 cells (ATCC), which are stably transfected with a CHE-Fc

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expressing construct and cotransfected with the neomycin resistance gene.[22] The cells grow as adherent monolayers and secrete the CHE-Fc chimera as a soluble protein. Best results are obtained by growing the cells in minimal essential medium $\alpha$ ($\alpha$MEM, Gibco) supplemented with fetal bovine serum (FBS), 2 m*M* L-Glutamine, 1 mg/ml geneticin (G-418 sulfate), and 100 U/ml penicillin/100 $\mu$g/ml streptomycin. While the stock culture to be carried indefinitely is maintained in 10% FBS and G-418, the CHE-Fc is ultimately harvested from medium containing 3–5% low IgG FBS (HyClone) and no added G-418 (see later). To optimize cell adherence, culture flasks may need to be first coated with 0.1 mg/ml poly-D-lysine (as per manufacturer's instructions). If so, flasks should then be washed once with PBS before cells are added.

Frozen cell stocks are initially thawed into $\alpha$MEM containing 20% FBS, L-glutamine, pen/strep, and no G-418. They are maintained as such for 48–72 h until the cells appear clearly adherent and healthy. This starter culture is then split 1:4–1:6 (depending on cell density) into T175 flasks, each containing a final volume of 50 ml of $\alpha$MEM, 10% low IgG FBS, L-glutamine, pen/strep, and G-418. Fifty to 100 ml of fresh medium containing no FBS or G-418 is added every 2–3 days as the medium appears to turn increasingly yellow. This usually proceeds for approximately 5–8 days, yielding flasks each with 250–300 ml of medium and a final FBS concentration near 3–5%. To maximize chimera yield, these confluent cells are then maintained an additional 7–10 days prior to harvesting, with no further perturbations. The longer one can maintain this culture, the higher the chimera yield will be, within the limits of maintaining medium pH and cell viability. Based on daily inspection, cells are harvested sooner rather than later if any evidence of peeling of cells off the flask and/or significant yellowing of the medium occurs.

*Suspension Cells.* More recently, the same stably transfected 293 cell line has been adapted to a protein-free medium CHO-S-SFM II (Gibco), allowing the cells to grow in bunches in suspension,[7] avoiding the contamination with bovine IgG during purification, and permitting the direct use of the medium without purification (see later). These cells are seeded into CHO-S-SFM 11 media containing 1 mg/ml geneticin and grown in T175 flasks. Fresh medium is added after approximately 7 days (depending on cell growth and condition of existing medium) and cells are then allowed to grow a further 3–4 days before harvesting the supernatant.

*Medium Harvest.* The spent medium from the adherent 293 cells just described is collected aseptically into 50-ml conical tubes and is spun at 2000 rpm for 15 min at 4° to remove any detached cells or cell debris. The supernatant is kept at 4° overnight and is then spun again to remove any remaining debris. This medium may then be frozen at −20° and pooled

for purification in batches as described later. Suspension cells in serum-free medium are handled differently. They are collected aseptically into 50-ml conical tubes and spun at 1000 rpm for 10 min at room temperature. Cell supernatants are then removed and respun to remove any remaining debris. The cleared supernatant is then pooled, concentrated approximately 100 × in Centriprep-YM30 filters (Amicon), and stored at 4° or frozen. This concentrated supernatant can typically be used without purification in a manner similar to the protein A-purified CHE-Fc from the adherent 293 cells (see later)

### Chimera Purification

*Reagents*

Protein A Sepharose (PAS) beads (Pharmacia)
Tris buffer, 1 *M*, pH 8.0
Sodium azide (Sigma)
Tris-buffered saline, pH 8.0
Poly-prep chromatography column (Bio-Rad)
Glycine–HCl buffer, 0.1 *M*, pH 3.0
Guanidine hydrochloride, 6 *M*

*Procedure*

Using 2–4 liters per batch, the frozen spent medium is thawed at 4°. This can take 48–72 h. If any debris persists, medium is spun again (2000 rpm, 15 min at 4°). For optimal binding of the Ig Fc tail of the chimeras to PAS, the pH of the medium is adjusted to ~8.0 using Tris buffer (the pH is usually already close to this range and does not require much adjustment). Being careful to avoid foaming, the medium is then split into 250-ml conical plastic tubes or placed in a 2.2-liter roller bottle(s) along with PAS beads at a concentration of approximately 100 $\mu$l PAS beads per 100 ml medium. To prevent microbial growth, 0.01% sodium azide is added. The tubes or bottles are incubated at 4° with constant, slow rolling for 48–72 h. The reason for doing this is that chimera loading onto PAS appears to be a slow process. Medium and beads are then spun at 1500 rpm rpm for 15 min at 4°, and the medium is decanted off the beads. At this point the medium may be discarded; however, there can be suboptimal chimera binding and one may find that further CHE-Fc can be ''rescued'' by repeat incubation of this decanted medium with PAS. Thus, we routinely save this decanted medium at 4° until an estimate of chimera yields is available. Using TBS to rinse the tubes or bottles, the beads are collected and transferred into a poly-prep chromatography column and maintained

at 4°. The column is then washed with several ml (3 bed volumes) of ice-cold TBS until the pink-tinged color of the medium clears completely. The CHE-Fc is then eluted with eight 1–1.5 bed volumes of cold glycine–HCl, pH 3.0. The eluate is allowed to flow slowly (1 drop/6 s) into 1.5-ml fractions each containing 100 $\mu$l of 1 *M* Tris buffer (pH 8.0). Each tube is mixed gently and adjusted further if needed to bring the pH back to neutral. CHE-Fc chimera yield and purity are checked on an 8% SDS–polyacrylamide gel under reducing conditions, running an aliquot of each eluate fraction separately, and then pooling those containing the chimera. The CHE-Fc runs at approximately 116–120 kDa under reducing conditions and at ~220 kDa under nonreducing conditions. Following gel analysis, one typically ends up pooling four to five eluate fractions, aiming for a final CHE-Fc concentration of ~0.4–0.5 mg/ml [some contaminating bovine IgG may be present so amounts are estimated by comparison to defined amounts of a standard protein (e.g. bovine serum albumin) run in the same gel and stained with Coommassie Blue]. If the preparation is too dilute, a Centricon-10 concentrator (Amicon Inc.) can be used to achieve the desired concentration. After elution, the column is washed with 3–4 bed volumes of TBS, cleaned by washing with 2 bed volumes of 6 *M* guanidine hydrochloride, and immediately reequilibrated with at least 5 bed volumes of TBS. These PAS beads may be reused several times if stored at 4–8° in 0.04% sodium azide.

Medium from serum-free suspension cells can also be subjected to the aforementioned purification procedure, as it contains other contaminating proteins secreted by the cells. However, for many applications, this medium can be used as is to avoid the variable losses and partial inactivation of CHE-Fc that does occur during the purification process.

*Storage and Stability.* Once prepared, the CHE-Fc chimera may be stored at −20° in aliquots for up to 9 months without any significant loss of activity. Multiple freeze/thaw cycles should be avoided. If more frequent use is expected, samples should be kept at 4° and will maintain good activity for 3–4 months. The CHE-FcD chimera, once DFP treated, may be stored similarly.

## Preparation of CHE-FcD

### *Reagents*

Diisopropyl fluorophosphate (DFP), 1 *M* and 100 m*M* stocks in isopropanol
NaOH, 1 *M*
Dialysis tubing, MWCO 12–14 kDa (Gibco BRL)

*Procedure*

A portion of the CHE-Fc preparation is treated with DFP stocks (note safety precautions given later) to a final concentration of 1 m*M* (typically by adding a 1% volume of the 100 m*M* stock). DFP treatment is allowed to proceed on ice for 45 min, and the preparation is dialyzed overnight against 1000 volumes of PBS at 4°. This seems to stabilize the preparation, presumably by eliminating breakdown products of DFP and the isopropanol. The final product is aliquoted and stored side by side with an equivalent CHE-Fc sample from the same original batch. Once prepared, CHE-Fc and CHE-FcD chimeras are analyzed for esterase activity and binding activity, respectively.

*Caution.* DFP is an extremely reactive and toxic chemical, even on skin contact. Store the stock solution in the refrigerator in a dessicator bottle marked ''Poison.'' All members of the laboratory should be aware of the presence and location of this bottle. Work should be performed in a well-functioning chemical hood with the appropriate protective clothing and in the presence of a second person. Double gloves, eye protection, and full sleeve coats must be worn. Keep the hood sash as low as possible and work on a plastic–backed disposable diaper. Dispose of contaminated tips in a small container containing 1 *M* NaOH. Use 1 *M* NaOH to clean up any possible points of contact or spills. Preparation of the DFP stock solution, as well as treatment of the chimera with DFP, must be performed under supervision by a second person with an atropine sulfate antidote (1 mg) loaded in a syringe at hand. If exposure to DFP is suspected, an intramuscular injection of atropine sulfate must be administered immediately and the individual taken to the emergency room with a call ahead regarding the potential need for more atropine. Because DFP degrades rapidly on contact with water, the 1 m*M* solution is not dangerous after 1 h on ice.

## Assay of Esterase Activity (CHE-Fc)

*Reagents*

Paranitrophenol (pnp) acetate (Sigma). Stock solution is 80 m*M* in acetonitrile
TBS

*Procedure*

The assay measures the hydrolysis of pnp-acetate by the CHE-Fc esterase. Because there is some spontaneous break down of the parent compound under the assay conditions, the rate of cleavage by the esterase is

compared with this background degradation. The assay is done in TBS with a 1 m*M* final concentration of pnp-acetate (made fresh). A known volume of a CHE-Fc preparation (e.g., 5 $\mu$l) is added and mixed well, and absorbance at 405 nm is measured in a spectrophotometer at time = 0 min and every 0.5 min for a total of 5 min. The CHE-FcD can serve as the negative control. For a reaction blank, use an equivalent volume of TBS and pnp-acetate. The assay may be performed in microtiter plates adapted for a plate spectrophotometer reader. A graph is plotted to measure the slope (rate of increase in absorbance per unit time). The extinction coefficient of pnp-acetate = 18,300 mol/liter.

(*slope of assay* − *slope of blank*) = umol/min for 5 $\mu$l of CHE-Fc ($\times$200 = units activity/ml)

Using this assay, we find that optimal batches of CHE-Fc have esterase activity in the range of 0.75–1.25 U/mg.

## CHE-FcD Binding Activity Assay

This assay is based on the avid binding of CHE-FcD to bovine salivary mucin (BSM), which is rich in 9-*O*-acetylated sialic acids, and the abrogation of such binding by pretreatment of the BSM with mild base (de-*O*-acetylation).

### *Reagents*

Nunc microwell plates (Nunc)
Bovine salivary mucin (Sigma)
De-*O*-acetylated (base treated) bovine salivary mucin (see later for preparation)
Coating buffer: 50 m*M* sodium carbonate/bicarbonate buffer, pH 9.5
PBS/0.1% Tween 20 (PBS-T)
2% BSA/PBS-T
Goat antihuman IgG (H+L)–AP conjugate (GxHuIgG-AP) (Bio-Rad)
CHE-Fc and CHE-FcD (200 mg/well)
Developer solution

### *Procedure*

*Base Treatment of BSM.* Fifty microliters of 1 *M* NaOH is added to 500 $\mu$l BSM (0.9 mg/ml) and incubated for 30 min at 37°. The reaction is neutralized with 50 $\mu$l 1 *M* HCl (pretitrated for accuracy), followed by the addition of 80 $\mu$l PBS.

Nunc plates are coated with 100 $\mu$l/well of BSM, base-treated BSM, or no BSM in coating buffer. The final concentration of BSM or base-treated BSM/well is 2 $\mu$g/ml.

After overnight incubation at 4°, the plate is washed three times with 200 μl/well of PBS-T, inverting briskly onto paper towels between washes. Wells are blocked with 100–200 μl/well 2% BSA/PBS-T for 2 h (and for up to 4–5 h) at room temperature. While blocking, the CHE-FcD and secondary antibody, GxHuIgG-AP, are precomplexed at 4° for at least 1 h in the dark in PBS-T. For each well needed, in a final volume of 100 μl/well, mix 200 ng CHE-FcD and the GxHIgG-AP secondary antibody at 1:1000 dilution. Precomplexing is required for optimal binding, as the chimera recognizes 9-*O*-acetyl sialic acids with high specificity but relatively low affinity. The exact ratio of CHE-FcD to GxHuIgG-AP must be optimized for each batch of secondary antibody used. Following blocking, the blocking solution is dumped from the plate and the prepared precomplexed chimera is added. Incubation of the plate at 4° for 2–4 h is followed by washing three times with PBS-T as described earlier. After adding 100 μl of developer/well, the plate is incubated at room temperature. A (yellow) color change is usually seen within 1–2 h. If not, the plate may be incubated at 4° overnight (lower temperature to prevent the reaction from proceeding too far) and read the following morning. The plate is read at 405 nm, and controls include secondary antibody alone, BSM +/− base, and coating buffer with no BSM.

## Use of CHE-Fc/FcD in Flow Cytometric Analyses

### *Reagents*

Goat F(ab′)2 antihuman IgG (Fc specific)-R-phycoerythrin-conjugated (GxHIgG-PE) (Caltag Laboratories)
CHE-Fc and CHE-FcD from the same batch
Staining buffer: 1% BSA/0.1% sodium azide in PBS
Wild-type C57B6 mouse blood

### *Procedure*

*Precomplexing.* Begin by precomplexing the CHE-Fc/FcD chimeras with a secondary antibody of choice. Precomplexing is required for optimal binding, as the chimera recognizes 9-*O*-acetyl sialic acids with high specificity but relatively low affinity. The exact ratio of CHE-FcD to GxHuIgG-conjugate has to be optimized and retitrated for each batch of secondary antibody. To titrate, use a fixed amount of CHE-Fc/FcD and various amounts of the secondary antibody and analyze by flow cytometry to find conditions for optimal staining in the cells of interest.

For each cell sample to be analyzed, in a final volume of 57 μl, add ~1 μg CHE-Fc or CHE-FcD, optimized dilution of GxHIgG-PE or secondary antibody of choice, and 50 μl of staining buffer (see earlier discussion).

This reaction can be scaled up for multiple samples and the precomplexing done in a single tube incubated on ice in the dark for 2 h.

*Positive Control: Mouse Red Blood Cells.* Murine erythrocytes carry 9-*O*-acetylated sialic acids on their surface and can be used both as a positive control for CHE-FcD staining in flow cytometry analyses and as an alternative to the BSM ELISA analysis of CHE-FcD-binding activity. A 2- to 3-$\mu$l sample of tail blood from a C57B6 mouse is collected, providing approximately $10^7$ erythrocytes $\mu$l. Cells are washed once in 10 ml of staining buffer and spun at 500 *g* for 3 min., and the pellet is resuspended in a further 10 ml staining buffer. A half million cells/tube are then aliquoted into FACS tubes (Falcon), respun, and the supernatant removed. The cells are now ready for staining as outlined next. Typically, at least 70–90% of erythrocytes stain positively with the CHE-FcD and negatively with CHE-Fc.

*Staining.* For staining with CHE-FcD, incubate washed, spun cells with the prepared precomplexed CHE-FcD *at 4°* for 2 h in the dark. Temperature differences are important if using CHE-Fc as a negative control, as this chimera does have some binding activity at 4° (because the esterase is less active). For a negative control with CHE-Fc, incubate washed, spun cells with the prepared precomplexed CHE-Fc *at room temperature* for 2 h in the dark. After staining, cells are then washed, spun, and resuspended in staining buffer per the specifications of the flow cytometer used for analysis. Note that in some instances where there is a very high density of 9-*O*-acetylation, the CHE-Fc control may not be completely negative.

When doing combined (double or triple) staining with additional antibodies/lectins, use volume, timing, and temperature conditions as practiced routinely for the cells being studied. When staining in combination, do any staining with precomplexed CHE-FcD first, and then after washing (once is sufficient), proceed with other antibodies. However, because the CHE-FcD may partially elute over time, do not allow long periods of secondary incubation.

*Esterase Treatment.* For some studies it may be necessary to remove 9-*O*-acetyl groups from cell surface sialic acids. Incubate the cell sample (0.5–1 million cells) in 50 $\mu$l of staining buffer with 10 $\mu$l (approximately 5 $\mu$g) of the CHE-Fc chimera at 37° for 1 h. Cells can then be washed and used as desired.

## Immunohistology

### *Reagents*

Superfrost +/+ slides (Fisher)
10% buffered formalin (Fisher)

Phosphate-buffered saline, pH 7.1
30% hydrogen peroxide (Fisher)
Mayer's hematoxylin (Sigma)
VIP substrate (Vector Labs).
Goat serum (Jackson Immunoresearch)
Bovine serum albumin (Sigma)
Peroxidase-conjugated donkey antihuman IgG (H+L, multiply absorbed) (Jackson ImmunoResearch)
Aqueous mounting media (Biomeda)
Murine frozen tissue sections (use mouse spleen as positive control in all experiments)
CHE-Fc/FcD

*Procedure*

Frozen sections of tissues are cut and air dried for at least 30 min at room temperature, followed by immersion in 0.03% $H_2O_2$/PBS for 30 min at room temperature (to quench endogenous peroxidase activity). Three PBS washes are then performed before immersion in fresh 10% buffered formalin for 30 min. After three more washes in PBS, the sections are overlaid with blocking buffer (1% BSA/PBS/10% normal goat serum) to block nonspecific binding and stored at 4° until ready to use. While blocking, the CHE-Fc (negative control) or CHE-FcD is precomplexed for at least 2 h end over end at 4° to the secondary antibody [peroxidase-conjugated donkey antihuman IgG (H + L)] in blocking buffer. This is done at a preoptimized ratio (typically 1:100 dilution, with each new batch of reagents requiring preoptimization using a positive tissue as a control). Each slide used requires precomplexed CHE-Fc/FcD at a final concentration of 4.5 $\mu$g/ml in a total volume of 200–500 $\mu$l, sufficient to cover the section(s), and is allowed to incubate for at least 2 h at 4°, followed by three PBS washes. Fresh VIP substrate is made according to the manufacturer's directions and is applied to the washed sections. This reaction is carried out in the dark for at least 10 min before again washing three times with PBS and counterstaining nuclei with Mayer's hematoxylin for 1 min. The sections are then coverslipped in aqueous mounting media and light microscopy is performed. An additional negative control should include sections containing secondary antibody alone (i.e., no CHE-FcD).

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