the oocytes. Omission of the heat-shock procedure resulted in junctional conductances not significantly different from those in untreated control oocytes. This is similar to the expression of β-galactosidase under the control of the heat-shock promoter [J. Ananthan, A. L. Goldberg, R. Voellmy, *Science* **232**, 522 (1986)].
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Selective Inactivation of Influenza C Esterase: A Probe for Detecting 9-*O*-Acetylated Sialic Acids

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The influenza C virus (INF-C) hemagglutinin recognizes 9-*O*-acetyl-*N*-acetylneuraminic acid. The same protein contains the receptor-destroying enzyme (RDE), which is a 9-*O*-acetyl-esterase. The RDE was inactivated by the serine esterase inhibitor diisopropyl fluorophosphate (DFP). [^{3}H]DFP-labeling localized the active site to the heavy chain of the glycoprotein. DFP did not alter the hemagglutination or fusion properties of the protein, but markedly decreased infectivity of the virus, demonstrating that the RDE is important for primary infection. Finally, DFP-treated INF-C bound specifically and irreversibly to cells expressing 9-*O*-acetylated sialic acids. This provides a probe for a molecule that was hitherto very difficult to study.

SIALIC ACIDS (NEURAMINIC ACIDS) can be *O*-acetylated at positions 4, 7, 8, or 9. We and others have demonstrated that these modifications are developmentally regulated (*1–3*), and that the addition of a single *O*-acetyl group can markedly affect the biological properties of the parent molecule (*4–6*). The biochemical study of *O*-acetylation of sialic acids in biological systems is fraught with many problems, especially loss and migration of *O*-acetyl groups during analysis (*7*). Since 9-*O*-acetyl-*N*-acetylneuraminic acid (Neu5,9Ac_2) is the preferred receptor for the hemagglutinin of influenza C (INF-C) (*8*) we reasoned that the virus could be used as a direct and specific probe for 9-*O*-acetylation. However, when INF-C is incubated with cells expressing Neu5,9Ac_2, an esterase [called the receptor-destroying enzyme (RDE)] causes a rapid and marked decrease in amount of the *O*-acetylation (*9*). We therefore sought ways to selectively block the esterase activity. As shown in Fig. 1, 1 m*M* diisopropyl fluorophosphate (DFP) completely inactivated the esterase. Phenylmethylsulfonyl fluoride (PMSF), another serine-active site inhibitor, was less effective, giving 25 to 50% inactivation at a 1 m*M* concentration.

After DFP treatment, the direct and specific binding of INF-C to cells could be demonstrated. Mouse erythrocytes, which are known to contain high levels of 9-*O*-acetylated sialic acids (*10*), were incubated with DFP-treated, biotinylated INF-C and then with fluorescent avidin. The INF-C–treated cells showed greater fluorescence (6.6-fold) than control cells (Fig. 2). This binding was completely blocked by bovine submaxillary mucin (which is rich in Neu5,9Ac_2) (Fig. 2) but not by an equivalent amount of bovine serum albumin. Thus, DFP-treated biotinylated INF-C is a specific probe for the presence of 9-*O*-acetylated sialic acids on cell surfaces.

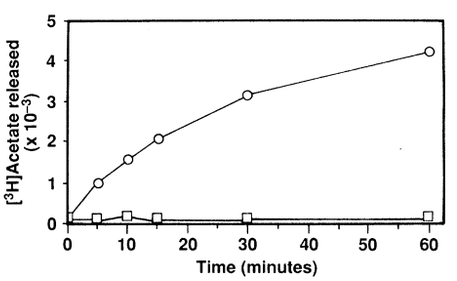

Fig. 1. Inactivation of INF-C esterase by DFP. INF-C (ATCC VR 104, C/Taylor/1233/47,5/80) was incubated with 1% isopropyl alcohol (IPA) or 1 m*M* DFP in 1% IPA for 30 minutes at 4°C. Treated virus was incubated with [^{3}H-acetyl] Neu5,9Ac_2 at 37°C. Reactions were stopped with an equal volume of a solution of 1*M* chloroacetic acid, 2*M* NaCl, and 0.5*M* NaOH, and contents of vials were added to 10 ml of scintillation cocktail which contained 20% isoamyl alcohol, 80% [0.5% 2,5-diphenyloxazole (PPO), 0.3% [1,4-bis-2(5-phenyloxazoyl)-benzene (POPOP) in toluene]. The principle of this assay and the details of the preparation of the substrate are as described (*20*). □, DFP-treated; ○, control.

These results also suggest that, although the hemagglutination and RDE activities of INF-C are both contained in the same glycoprotein and recognize the same structure (Neu5,9Ac_2), they do not share the same binding site. To confirm this, we studied the hemagglutination properties of the treated virus. Hemagglutination activity was not blocked by DFP-treatment of INF-C (Fig. 3). When the cells were warmed to 22°C, esterase activity caused the predicted loss of hemagglutination in the controls and the PMSF-treated sample. However, hemagglutination was preserved in the DFP-treated sample, and persisted for several days at 4°C. Thus, the binding of DFP to the serine active site of the esterase did not block the hemagglutination function; indeed, it made it stable rather than rapidly reversible.

To confirm the binding of DFP to the glycoprotein, we labeled INF-C virions with [^{3}H]DFP and then examined the virus proteins by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Previous studies have indicated that the activated influenza C glycoprotein (gpII)

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has an approximate molecular mass (M_r) of 82,000 (82K), and is derived by tryptic cleavage from a precursor (gpI) of approximately 105K (*11*). GpII is composed of two disulfide-bonded subunits, gp65 (HA I), and gp30 (HA II). Under nonreducing conditions the major [^{3}H]DFP-labeled peptide had an M_r of 89K (corresponding to gpII) (Fig. 4). In the presence of a reducing agent, the size of the peptide shifted to 63K (corresponding to gp65). There was a minor band of 113K prior to reduction, and a less distinct band at 92K after reduction, which may represent a small amount of gpI. The gp30 band was not labeled, thus localizing the DFP binding site to gp65 (HAI).

Review of previously described serine protease and esterase active sites indicates that a Gly-X-Ser-X-Gly sequence is consistently found, presumably because of a common origin from a primordial precursor (*12, 13*). Upon examination of complementary DNA–derived amino acid sequences of the INF-C glycoprotein from two independent isolates (*11, 14*), no such consensus sequence was found in gp65. Thus, it appears that the serine active site of the INF-C 9-*O*-acetyl-esterase must be a novel one. Since

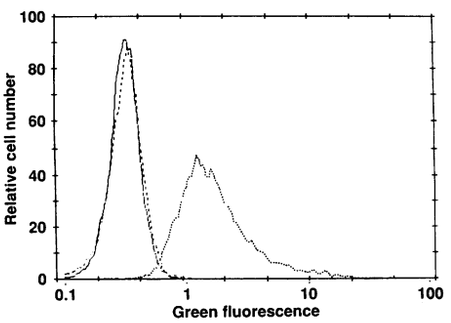

Fig. 2. Direct and specific binding of DFP/INF-C to mouse erythrocytes. INF-C was incubated at 4°C for 30 minutes with Sulfo-NHS-Biotin (1 mg/ml; Pierce Chemicals) in phosphate-buffered saline (PBS) with 1 m*M* DFP. Virions were separated from unbound biotin and free DFP by ultracentrifugation at 100,000*g* through 0.25*M* sucrose, 10 m*M* sodium phosphate, *p*H 7.2, for 30 minutes at 4°C. Recovery of virus ranged from 20 to 40%, as measured by the esterase activity of a parallel aliquot of non–DFP-treated virus. BALB/c mouse erythrocytes (2×10^6) suspended in 0.02 ml PBS were incubated with an equal volume of PBS (– – –); DFP-treated INF-C (····); or DFP-treated INF-C plus 10 μg of bovine submaxillary gland glycoproteins (*5*) (——) for 30 minutes on a rotary mixer at 4°C. Samples were washed three times in PBS and then incubated with 0.1 ml 1:50 fluorescein isothiocyanate (FITC)-avidin (E-Y Labs) for 30 minutes on the 4°C rotary mixer and washed three times with PBS. Samples were suspended at 2×10^6 cells per milliliter and run on Cytofluorograf IIS flow cytometer (Ortho Diagnostic Systems, Inc., Westwood, MA). A total of 5000 cells were analyzed per sample. The FITC fluorescence was excited at 488 n*M* and the emissions were collected in the 510- to 580-nm bandwidth.

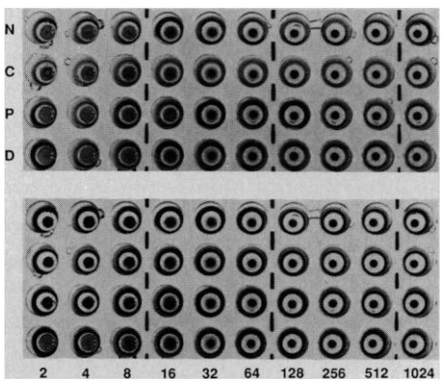

Fig. 3. Effect of DFP upon hemagglutination. INF-C was incubated for 30 minutes at 4°C with PBS, 1% isopropyl alcohol (IPA), 1 m*M* PMSF in 1% IPA, or 1 m*M* DFP in 1% IPA. A portion (0.025 ml) of each virus preparation was added to the first well containing 0.025 ml of PBS, *p*H 7.2, and serially diluted. Chicken erythrocytes (0.025 ml; packed cell volume, 1.5%) were added to each well. The plate was photographed after 1 hour at 4°C (upper panel), incubated for 2 hours at room temperature, and rephotographed (lower panel). N, no treatment; C, control; P, PMSF; D, DFP. The hemagglutinin titer is shown at the bottom.

the INF-C glycoprotein has clear homology to the INF-A and INF-B hemagglutinins (*11, 14*), it is conceivable that the origin of this esterase activity was by convergent rather than divergent evolution.

The third function of the INF-C glycoprotein is low *p*H–mediated membrane fusion, which allows penetration of the host endosomal membrane. We tested the fusion activity of DFP-treated INF-C virions by hemolysis of mouse erythrocytes (*15*), and found it to be unchanged from that of control virus. This is consistent with the proposed location of the fusion peptide at the amino terminus of gp30 (*11*), which is not modified by DFP.

The HA and fusion activities of influenza viruses are clearly involved in the primary infection process. However, in spite of some experiments with temperature-sensitive INF-A mutants (*16–18*) the role of the RDE (which in INF-A and INF-B is a neuraminidase) in this process remains controversial. Since DFP-treated INF-C virions have intact receptor and fusion functions, we used them to determine if the RDE (esterase) has any role in infectivity of this virus. INF-C virus preparations were treated with DFP or isopropanol solvent alone, pelleted by ultracentrifugation, serially diluted, and injected into embryonated eggs (*19*). The recovery of virus was monitored by assay of the esterase. We found that DFP treatment caused a 100-fold decrease in the titer of infectivity [infection established with 1:10,000 dilution American Type Culture Collection stock with titer $10^{9.5}$ EID_{50} (median effective infectious dose) per 0.2 ml for control virus but only with 1:100 dilution for DFP-treated virus]. Although some virus could be recovered from eggs injected with concentrated DFP-treated preparations, the esterase activity remained sensitive to DFP. This demonstrates that these viruses are the progeny of a few that escaped inactivation, rather than variants with a DFP-resistant active site. Taken together, these data suggest that the RDE must play an important role in the establishment of a primary infection by INF-C.

Study of the cell biology and biochemistry of 9-*O*-acetylation of sialic acids has been greatly limited because of the technical difficulties of analysis of these labile ester groups. We have demonstrated that DFP treatment stabilizes the 9-*O*-acetylated sialic acid–specific binding of INF-C. Thus, the

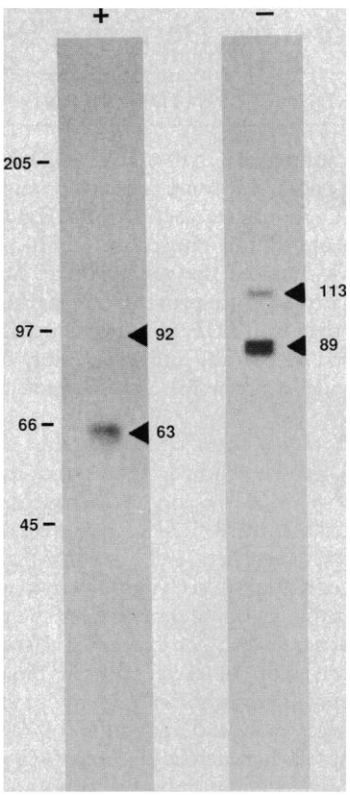

Fig. 4. SDS-PAGE of [^{3}H]DFP-labeled INF-C. INF-C (1 mg of protein) was incubated in 0.2 ml of PBS with 20 μCi of [1,3-^{3}H]DFP (35.3 mCi/mg, Amersham) for 30 minutes at 4°C. Labeled virus was separated from unbound DFP by ultracentrifugation at 100,000*g* for 1 hour through a 0.25*M* sucrose, 10 m*M* sodium phosphate solution, *p*H 7.2. The pelleted virus was resuspended in PBS and two-thirds were solubilized in 2% SDS, 10% glycerol, 65 m*M* tris HCl, *p*H 6.8 with (+) and without (–) 1% 2-mercaptoethanol. Samples were studied by SDS-PAGE (10% gel) as described (*21*) with the exception that the separating gel buffer was at *p*H 7.8. The gel was run at 4°C for 6 hours at 20 mamp, fixed, impregnated with Enhance (NEN), and exposed for 4 days at –70°C in the presence of XAR film (Eastman Kodak). The M_r ($\times 10^{-3}$) is shown.

intact virus and/or the purified hemagglutinin can now be used as a powerful tool for the direct investigation of 9-*O*-acetylation in intact cells and tissues.

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Regulation of *bcl*-2 Proto-Oncogene Expression During Normal Human Lymphocyte Proliferation

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The *bcl*-2 and c-*myc* proto-oncogenes are brought into juxtaposition with the immunoglobulin heavy chain locus in particular B-cell lymphomas, resulting in high levels of constitutive accumulation of their messenger RNAs. Precisely how the products of the *bcl*-2 and c-*myc* genes contribute to tumorigenesis is unknown, but observations that c-*myc* expression is rapidly induced in nonneoplastic lymphocytes upon stimulation of proliferation raise the possibility that this proto-oncogene is involved in the control of normal cellular growth. In addition to c-*myc*, the *bcl*-2 proto-oncogene also was expressed in normal human B and T lymphocytes after stimulation with appropriate mitogens. Comparison of the regulation of the expression of these proto-oncogenes demonstrated marked differences and provided evidence that, in contrast to c-*myc*, levels of *bcl*-2 messenger RNA are regulated primarily through transcriptional mechanisms.

NONRANDOM CHROMOSOMAL TRANSlocations are found in a variety of leukemias and lymphomas, suggesting that these specific genetic changes impart a selective growth advantage. In some cases these translocations involve a known proto-oncogene and result in the "activation" of the gene through alterations either in the regulation of its expression or in its coding sequences (*1*). Perhaps the best studied examples of translocations associated with a lymphoid neoplasm are those involving the c-*myc* proto-oncogene in Burkitt lymphoma, wherein the c-*myc* gene on chromosome 8 becomes juxtaposed to sequences in the immunoglobulin heavy [t(8;14)], κ [t(2;8)], or λ [t(8;22)] chain loci. Translocations involving the c-*myc* gene result in constitutive accumulation of high levels of c-*myc* messenger RNA (mRNA) through mechanisms that are poorly understood but that probably involve enhancer-like elements within the transcriptionally active immunoglobulin loci in these B-cell neoplasms (*2*).

In addition to translocations involving c-*myc* on chromosome 8 in Burkitt lymphoma, other B-cell neoplasms harbor translocations involving immunoglobulin loci, such as the t(11q13;14q32) found in a variety of acute and chronic B lymphocytic leukemias and the t(14q32;18q21) seen in most follicular B-cell lymphomas (2). Although none of the known proto-oncogenes appears directly involved in these translocations, it has been postulated that such genes may reside at 11q13 and 18q21 (termed *bcl*-1 and *bcl*-2, respectively, for B-cell lymphoma/leukemia 1 and 2), and that they may become activated by analogy to the c-*myc* gene in Burkitt lymphoma.

By means of chromosomal walking techniques, the *bcl*-2 gene has recently been cloned and sequenced (*3, 4*). The predicted products of human *bcl*-2 appear to be 26-kD (*bcl*-2-α) and 22-kD (*bcl*-2-β) proteins that (i) differ in their carboxyl termini as a result of alternative splice site selection; (ii) lack transmembrane, leader, or kinase domains; and (iii) share no significant homology with other proteins whose sequences are known (*4*). In all lymphomas with t(14q32;18q21) thus far examined, the rearranged *bcl*-2 gene is expressed at high levels and shows no evidence of gross alterations in its coding sequences (*4, 5*). Hence, aberrant expression of the *bcl*-2 gene, rather than an abnormal gene product, most likely accounts for any selective growth advantage that the t(14q32;18q21) may impart.

Given the possibility that expression of the *bcl*-2 gene may contribute to the altered growth characteristics of malignant lymphocytes harboring a t(14;18), we wondered whether expression of this gene occurs during normal lymphocyte proliferation. Several proto-oncogenes, including c-*myc*, become expressed in normal lymphocytes after stimulation with appropriate mitogens (*6, 7*). We therefore sought to determine whether the *bcl*-2 gene is expressed in these cells. Though oncogenic activity has yet to be demonstrated for *bcl*-2, we henceforth refer to the normal form of this gene as a proto-oncogene.

Figure 1A shows Northern blot data derived from human peripheral blood lymphocytes (PBL) that had been stimulated for various times with the mitogenic lectin phytohemagglutinin (PHA). Because levels of ribosomal RNA (rRNA) increase in PBL after stimulation with PHA, we compared equal amounts of total cellular RNA rather than RNA from equal numbers of cells. Accumulation of 8.5- and 5.5-kb mRNAs for *bcl*-2-α and of a less abundant 3.5-kb mRNA for *bcl*-2-β rose from undetectable to maximal levels within 6 to 14 hours after stimulation of PBL with PHA. These three mature transcripts result from alternative polyadenylation and splice site selections

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