JOURNAL OF VIROLOGY, Apr. 1985, p. 53–57
0022-538X/85/040053-05$02.00/0

Vol. 54, No. 1

Cytotoxic T-Lymphocyte Reactivity with Individual Sendai Virus Glycoproteins

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Received 4 September 1984/Accepted 19 December 1984

Liposomes were constituted with affinity-purified Sendai virus glycoproteins HN and F and phosphatidylcholine (PC) or phosphatidylethanolamine: phosphatidylserine (PEPS). The glycoprotein-bearing recombinant vesicles (RV) were used to modify the surface of P815 mastocytoma cells (H-2^d) or EL4 lymphoma cells (H-2^b). The cells treated with HN-F-PCRV, HN-PEPSRV, or F-PEPSRV were shown by surface immunofluorescence to retain antigen for at least 2 h at 37°C after treatment. The modified cells were used in cytotoxicity assays with effector spleen cells from either DBA/2 (H-2^d) or C57BL/6 (H-2^b) immunized by inoculation of active Sendai virus. Cells modified by treatment with HN/F-PCRV showed susceptibility to cytolysis similar to that in actively infected cells. Cells modified with HN-PEPSRV or with F-PEPSRV were also susceptible. The sum of reactivities of the anti-HN component and the anti-F components was close to that seen with HN- and F-bearing targets. Syngeneic but not allogeneic target cells expressing Sendai virus glycoproteins were bound and lysed by the effector cells, which was expected if the interactions were major histocompatibility complex restricted. The activity was attributed to cytotoxic T lymphocytes, since it was depleted by treatment with anti-Thy 1.2 antibody and complement.

Immune responses to paramyxovirus infections are believed to be directed mainly against the glycoprotein peplomers of the virus envelope. Antibodies against viral glycoproteins inhibit virus adsorption and penetration and, therefore, protect against infection (3). It is generally accepted that cell-mediated immunity is the first line of specific immune host defense in enveloped virus infection and that it is directed against cells expressing viral glycoproteins and syngeneic major histocompatibility complex antigens on their surfaces (4, 5, 10). Previous studies have shown that HN and F envelope glycoproteins of Sendai virus, a prototype of paramyxoviruses, can be incorporated into liposomes to stimulate both primary and secondary cytotoxic T lymphocytes (CTL), whereas liposomes containing HN and inactive F glycoproteins or either glycoprotein alone fail to generate CTL response (6, 12). It is not yet understood, however, whether HN or F or both are recognized as the molecular structures upon which cytolytic immune cells act. Furthermore, the contribution and the relative importance of each glycoprotein to such recognition need to be investigated. In this report, we describe a procedure in which HN and F were incorporated separately and together into tumor cell membranes and the use of these modified cells as targets to detect cell-mediated cytotoxicity against each glycoprotein. The requirement for syngeneic target cells and the identity of the effectors that were generated in the primary response to immunization by active virus were determined.

MATERIALS AND METHODS

Virus. The Cantell strain of Sendai virus was grown in the allantoic cavity of 10-day-old embryonated eggs. The allantoic fluid was harvested and clarified by centrifugation at 3,000 × *g* for 30 min at 4°C, and the virus was pelleted at 60,000 × *g* for 2 h at 4°C. Further purification was carried out by sedimenting the virus to equilibrium in a 15 to 60% (wt/wt) sucrose gradient as described by Haywood (8). The protein concentration as determined by the method of Bradford (2) was adjusted to 1 mg/ml in 10 mM phosphate buffer (pH 7.2).

Affinity chromatographic preparation of HN and F glycoproteins. The virus particles were disrupted by adding the nonionic detergent β-D-octylglucoside (Calbiochem-Behring, La Jolla, Calif.) to a final concentration of 1% (33 mM). The detergent-virus suspension was mixed and incubated at room temperature for 1 h followed by centrifugation at 100,000 × *g* for 30 min at 4°C to remove intact virus and other nonsolubilized components. The supernatant contained HN, F, viral lipids, and β-D-octylglucoside.

Monospecific antibodies to HN and F were coupled separately to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N.J.) as described in the package insert, except that 33 mM β-D-octylglucoside was present in the starting material. Anti-HN and anti-F sera were absorbed several times on F-Sepharose and HN-Sepharose columns, respectively, to attain monospecificity. Absorbent beads for the columns were prepared with HN separated on DEAE-Biogel and F separated on hydroxyapatite (12, 15). While both of the glycoproteins used for absorption had low levels of contaminating protein, they effectively removed unwanted antibody. The monospecificity of the immunoglobulin G (IgG) preparations used for the affinity columns was determined by western blotting against whole Sendai virus. HN and F were separated by running the supernatant through an anti-HN-Sepharose column. The collected effluent was run through an anti-F-Sepharose column. In all cases, the glycoprotein molecules were eluted by 3 M KSCN (pH 7.2). KSCN was removed by dialysis against phosphate buffer.

Reconstitution of glycoproteins into RV. Recombinant vesicles (RV) were prepared by a detergent-dialysis method (1) with purified HN or F and a 1:1 mixture of phosphatidylethanolamine-phosphatidylserine (PEPS) (Avanti Biochemicals, Birmingham, Ala.). HN and F were incorporated

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together into RV with phosphatidylcholine (PC) (GIBCO Laboratories, Grand Island, N.Y.). The protein-bearing RV were isolated by centrifugation (60,000 × *g* for 18 h at 4°C) through 10 to 45% sucrose gradients for HN/F-PCRV or 0 to 45% sucrose gradients for HN-PEPSRV and F-PEPSRV. Fractions containing HN/F-PCRV were dialyzed against phosphate buffer, and those containing HN-PEPSRV and F-PEPSRV were dialyzed against 0.01 M Tris (pH 7.4)–5 mM $CaCl_2$–0.1 mM dithiothreitol. The protein concentration of RV preparations was adjusted to 100 μg/ml.

Cell lines. P815 mastocytoma tumor cells from DBA/2 (H-2^d) mice and EL4 lymphoma cells from C57BL/6 (H-2^b) mice were maintained by weekly transfer of 10^7 ascites cells in mice of the strain of origin for 4 weeks. The cells were then grown as a stationary cell suspension in RPMI 1640 medium (GIBCO) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid) buffer, 25 mM $NaHCO_3$, and 25 μg of gentamicin per ml (RPMI-FBS). The pH of the medium was maintained at 7.4, and the cells were passaged every other day.

Preparation of cytotoxic lymphocytes. DBA/2 (H-2^d) and C57BL/6 (H-2^b) female mice (Jackson Laboratory, Bar Harbor, Maine) were 6 to 12 weeks old when each was immunized by intraperitoneal inoculation of 200 μg of purified Sendai virus. The spleens were removed 7 to 10 days later, and the single-cell suspensions were prepared. After filtration through nylon mesh, erythrocytes were lysed with ACK buffer (0.16 M NH_4Cl, 0.01 M $KHCO_3$ [pH 7.2]), and the cells were washed twice with Hanks balanced salt solution. The cell concentration was adjusted to 2×10^7 cells per ml in RPMI-FBS.

Target cells. P815 and EL4 cells were passaged and, after 24 h, washed and treated with protein-bearing RV. Measured amounts (protein weight) of HN-F-PCRV, HN-PEPSRV, or F-PEPSRV were incubated with 5×10^5 cells at 0°C for 15 min and transferred to 37°C for 45 min. After one washing, they were suspended in 0.2 ml of RPMI-FBS and labeled with 50 μCi per 10^6 cells of $Na_2{}^{51}CrO_3$ (New England Nuclear Corp., Boston, Mass.) at 37°C for 45 min in a shaking water bath. Control cells were labeled in the same manner. Cells were washed twice with RPMI-FBS and counted, and the suspension was adjusted to 2×10^5 cells per ml. Control targets were Sendai virus-infected cells (18 to 24 h postinfection; multiplicity of infection, 10) and untreated cells.

Measurement of the amount of HN and F expressed on cell surfaces. The amount of cell surface antigen was measured with an enzyme immunoassay. P815 cells were treated with HN-PEPSRV, F-PEPSRV, or UV-irradiated Sendai virus at 0°C for 15 min followed by 37°C for 45 min. Cells were washed three times with Hanks balanced salt solution and incubated with a 1:20 dilution of the anti-HN or anti-F serum at 0°C for 1 h. Portions of actively infected P815 cells (24 h; multiplicity of infection, 10) and uninfected cells were also treated with the antisera. After incubation and three washes in Hanks balanced salt solution, cells were placed in plastic tubes previously treated with 1% bovine serum albumin in carbonate-bicarbonate buffer (pH 9.0). Horseradish peroxidase-conjugated goat anti-rabbit IgG (H- and L-chain specific; Cappel Laboratories, Cochranville, Pa.) diluted 1:1,000 in phosphate-buffered saline with 1% bovine serum albumin was added to the cells, and they were further incubated at 0°C for 1 h. The contents of the tubes were washed three times with Hanks balanced salt solution, and the substrate *o*-phenylenediamine was added at a concentration of 70 mg/100 ml of substrate buffer. Tubes were placed at 37°C for 30 min, quickly cooled on ice, and centrifuged at 4°C. The optical density (OD) of the supernatant was read in an EIA Reader (Bio-Tek Instruments, Burlington, Vt.) at 490 nm. OD values were corrected by subtracting the average of triplicate readings of supernatants from the uninfected P815 cells.

Cytotoxic assay. The assays were performed in triplicate in 96-well Limbro microtiter plates as described previously (13). Briefly, 2×10^4 target cells per 0.1 ml were placed in each well, and the proper number of effector cells in 0.1 ml was added. Effector-to-target ratios of 100, 75, 50, and 25 were used to determine the experimental release unless otherwise stated. To determine maximum release, 5×10^5 cells of each target type were lysed in 1 N HCl, whereas to determine spontaneous release, 5×10^5 cells were placed in medium alone. Plates was centrifuged at 40 × *g* for 5 min, covered, and incubated at 37°C for 4 h in 7% CO_2. The test plates were then centrifuged at 650 × *g* for 10 min, and 0.1 ml of cell-free supernatant from each well was assayed for released radioactivity in a Packard Autogamma Scintillation Spectrometer (Packard Instrument Co., Inc., Rockville, Md.), and the specific release of the isotope was determined by the formula: percent specific release = [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100.

Cold-target competitive inhibition. P815 and EL4 cells were treated with HN-F-PCRV and labeled with ^{51}Cr as described. Another population of the cells was treated with HN/F-PCRV but was not labeled. The chromium release assay was performed as described before except that (i) the effector-to-target ratio was 50, (ii) the number of labeled targets was the same but was contained in a 50-μl volume, (iii) cold target cells in 50-μl volumes were mixed with effector cells (100 μl) for 20 min at 37°C before the addition of radiolabeled target cells, and (iv) a cold inhibitor-to-hot target ratio of 2:1 was used.

Treatment of effectors with anti-Thy 1.2 monoclonal antibody and complement. Effector cells were adjusted to 2×10^7/ml. One-third of the effectors were centrifuged and suspended in a volume similar to that of the original in supernatant from hybridoma culture (clone 30-H12) containing rat monoclonal antibodies (IgG2b) specific for Thy 1.2 antigen (11), and rabbit complement was added to a final dilution of 1:15. The rest of the effector cells were divided into three parts to be used as controls and were sedimented. One part was suspended in the hybridoma supernatant, the second was suspended in medium and a final dilution of 1:15 of rabbit complement, and the third was suspended in medium only. All were incubated for 45 min in a shaking water bath, washed once with cold medium at 4°C, and adjusted to 2×10^7 cells per ml. Targets were P815 and EL4 cells treated with HN-F-PCRV. All target cells were labeled with ^{51}Cr and used with the treated effectors in the chromium release assay described above.

RESULTS

Purification of viral glycoproteins. Figure 1 shows a sodium dodecyl sulfate-polyacrylamide gel electrophoresis preparation of the starting supernatant material and the affinity-purified HN and F glycoproteins. The 3 M KSCN eluate from the anti-HN column contains HN and is free of F, whereas the eluate from the anti-F column contains only F.

Interaction of protein-bearing RV with cell surfaces. RV (5 to 10 μg protein weight) were incubated with 5×10^5 P815 or EL4 cells for 15 min at 0°C and for 45 min at 37°C. The

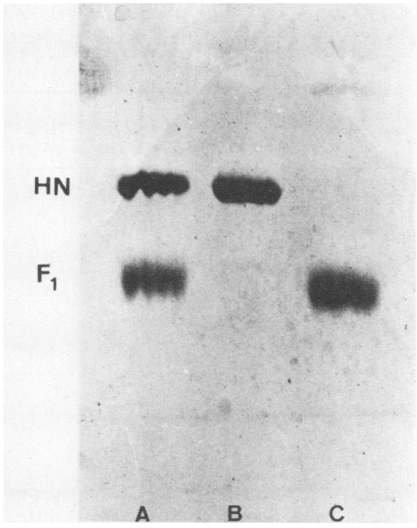

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis preparation (silver stain) of (A) supernatant containing HN and F, (B) 3 M KSCN eluate from an anti-HN Sepharose column, and (C) 3 M KSCN eluate from an anti-F Sepharose column.

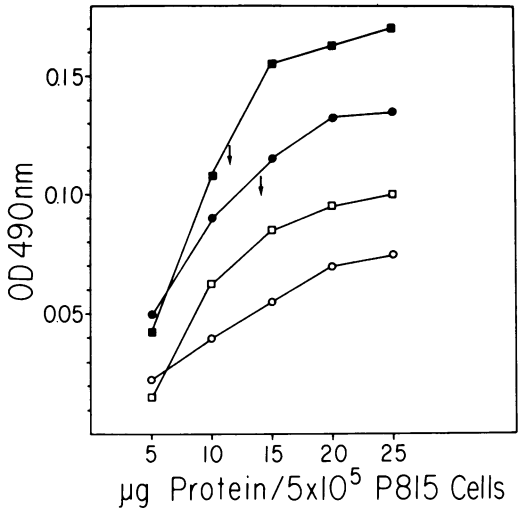

FIG. 2. Determination of the amount of protein in RV which, when introduced to P815 cells, results in the expression of HN and F comparable with that in natural infection. Cells (5×10^5) actively infected with Sendai virus showed an OD_{490} value of 0.122 when tested with anti-F and an OD_{490} value of 0.109 when tested with anti-HN as indicated by the arrows. F-PEPSRV-modified cells tested with anti-F (■), HN-PEPSRV-modified cells tested with anti-HN (●), UV-inactivated Sendai virus-modified cells tested with anti-F (□), and UV-inactivated Sendai virus-modified cells tested with anti-HN (○) are shown.

presence of the glycoproteins on the cell surfaces was demonstrable by direct immunofluorescence with monospecific fluorescein isothiocyanate-conjugated anti-HN and tetramethyl rhodamine isothiocyanate-conjugated anti-F. The glycoproteins were demonstrable after incubation at 37°C for 2 h. Table 1 illustrates the retention of the antigens.

Determination of the amount of HN and F expressed on treated cells. We conducted an experiment to determine the amount of glycoprotein needed to produce target cells displaying approximately the same amount of antigen as virus-infected cells. P815 cells were treated with various amounts (protein weight) of HN-PEPSRV, F-PEPSRV, or UV-irradiated Sendai virus for 15 min at 0°C and for 45 min at 37°C. The amount of surface antigen expressed on the surface of the treated cells and actively infected cells was measured by the enzyme immunoassay method described above. Figure 2 shows the results of this experiment. Cells expressed increasing amounts of surface antigen when treated with 5, 10, and 15 μg of HN-PEPSRV or F-PEPSRV (protein weight). At larger amounts, the increase in measured activity was less. UV-irradiated virus caused the expression of both HN and F but to a lesser degree. The OD value for F on the surface of actively infected cells was 0.122, OD and for HN it was 0.109. The equivalent amount of F or HN introduced by the RV resulted from treatment of the cells with 10 to 15 μg of glycoprotein.

Lysis of glycoprotein-bearing cells by CTL. The susceptibility of the glycoprotein-bearing cells to CTL-mediated lysis was tested in a ^{51}Cr-release assay with effector cells from mice immunized by intraperitoneal inoculation of 200 μg of active Sendai virus. Effector lymphocytes were prepared from DBA/2 (H-2^d) and C57BL/6 (H-2^b) mice and tested against syngeneic and allogeneic targets. Figure 3 shows the results of testing the H-2^d effectors against P815 cells modified by insertion of HN-F, HN, or F. They were also tested against Sendai virus-infected cells. The effectors caused similar lysis of the HN-F-bearing cells and the Sendai-infected cells at all effector-to-target ratios. They caused less lysis of the F-bearing cells and still less of the HN-bearing cells. The magnitude of the lysis of F-bearing cells added to that of the HN-bearing cells was close to the total lysis of the HN-F-bearing cells and the Sendai-infected target cells. When tested against allogeneic H-2^b targets, the effectors failed to cause significant cytolysis. Syngeneic spleen cells from nonimmune mice similarly failed to lyse the targets to any significant degree. Similar results were obtained when H-2^b effectors from C57BL/6 mice were tested against syngeneic and allogeneic targets.

Cold-target inhibition of cytolysis. Table 2 shows the results of cold-target inhibition experiments with various unlabeled cells. In the case of P815 cells, significant inhibition of HN-F-modified cells was effected by unlabeled HN-F-bearing P815 cells and Sendai virus-infected P815

TABLE 1. Surface fluorescence of P815 cells treated with protein-bearing RV[a]

Stain	Cell treatment								
	HN/F-PCRV incubated at:			HN-PEPSRV incubated at:			F-PEPSRV incubated at:		
	0°C for 1 h	37°C for 1 h	37°C for 2 h	0°C for 1 h	37°C for 1 h	37°C for 2 h	0°C for 1 h	37°C for 1 h	37°C for 2 h
Anti-HN	++++	+++	+++	++++	+++	+++	−	NT[b]	NT
Anti-F	++++	+++	+++	−	NT	NT	++++	+++	+++

[a] Percentage of cells showing surface fluorescence: ++++, 80 to 100%; +++, 50 to 80%; ++, 25 to 50%; +, 1 to 25%; −, <1%.
[b] NT, Not tested.

cells but not by the allogeneic inhibitors. Similarly, inhibition of specific lysis of HN-F-modified EL4 cells was achieved by syngeneic targets but not by allogeneic targets. These experiments confirmed the requirements for both the syngeneic target cells and specific antigen expression for effector-target cell interactions.

Abolition of specific lysis by treatment of effectors with anti-Thy 1.2 and complement. Cytotoxicity assays with effectors from immunized DBA/2 mice treated with anti-Thy 1.2 and complement were conducted on HN-F-modified P815 targets. Antibody or complement alone failed to abolish cytolysis, but together they removed the active cells.

DISCUSSION

We have previously shown that cells modified by the insertion of HN-F by vesicles formed with viral lipids can be lysed by naturally cytotoxic cells (1). In the present report, we describe the use of cells modified by insertion of HN-F and each glycoprotein separately as targets for CTL. The modification of the target cells was effected by incorporating the glycoproteins into vesicles composed of purified phospholipid, either PC or PEPS. In other work (M. Al-Ahdal, T. Abidi, and T. D. Flanagan, unpublished data), we described the characteristics of interaction of glycoprotein-bearing RV with the surfaces of P815 and EL4 cells and the subsequent behavior of the glycoproteins in the cell membrane. Most significantly for the current work, we observed that HN-PCRV eluted from the surface of the treated cells and that F-PCRV did not appear to interact with the cell surface at all. However, with HN-PEPSRV and F-PEPSRV in the presence of 5 mM $CaCl_2$, each glycoprotein was inserted into the membrane and retained for up to 2 h. HN/F-PCRV effected the insertion of both glycoproteins and their retention for up to 2 h. The results of these investigations led us to select HN/F-PCRV, HN-PEPSRV, and F-PEPSRV to modify the cell surfaces of potential targets for CTL.

It was necessary to introduce amounts of viral glycoprotein equivalent to those expressed on actively infected cells. Enzyme immunoassays conducted on modified cells suggested saturation of the available surface when cells were treated with amounts greater than 20 to 25 μg per 5×10^5 cells. Measurement of F and HN on actively infected cells indicated that equivalent expression could be achieved by treatment with 10 to 15 μg of F-PEPSRV or HN-PEPSRV. Treatment of targets was done with this amount of viral glycoprotein.

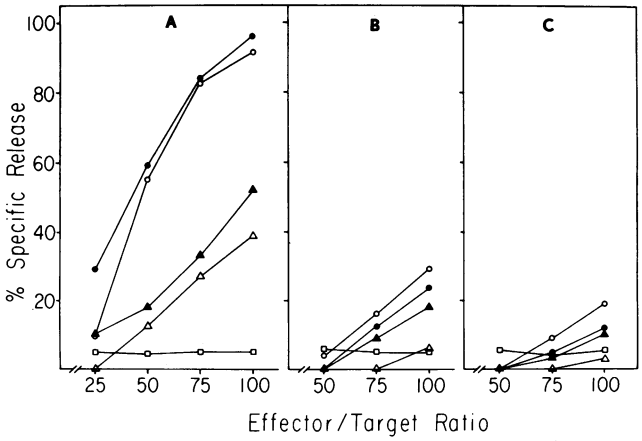

FIG. 3. ^{51}Cr release from P815 target cells infected with Sendai virus (○), modified with HN/F-PCRV (●), HN-PEPSRV (△), F-PEPSRV (▲), or untreated (□) effected by spleen cells from immunized syngeneic DBA/2 (H-2^d) mice (panel A), immunized allogeneic C57BL/6 (H-2^b) mice (panel B), and nonimmunized syngeneic mice (panel C).

TABLE 2. Competitive inhibition of specific lysis of labeled target cells by unlabeled syngeneic or allogeneic cells

Immunized donor strain	^{51}Cr-labeled targets	Inhibitors (% specific lysis) with[a]: No inhibitors	P815 + HN/F-PCRV	P815 (SV-infected)	EL4 + HN/F-PCRV	EL4 (SV-infected)
DBA/2 (H-2^d)	P815 + HN/F-PCRV	56.5	5.6	5.6	56.3	50.9
	P815	5.2	NT[b]	NT	NT	NT
C57BL/6 (H-2^b)	EL4 + HN/F-PCRV	55.5	53.1	54.6	5.6	5.9
	EL4	2.2	NT	NT	NT	NT

[a] Inhibitor-to-target ratio was 2:1.
[b] NT, Not tested.

The generation of CTL was carried out by intraperitoneal injection of active Sendai virus. We attempted to use UV-inactivated virus to evoke CTL; however, doses of up to 500 μg intraperitoneally did not produce significant activity in spleen cells harvested up to 14 days postinoculation. Active virus was effective in evoking CTL that were detectable 7 to 10 days after inoculation. This experience was similar to that reported by Kibler et al. (9).

Our results indicate that CTL are generated that recognize the HN glycoprotein as well as the F protein. The total amount of CTL activity appears to be the sum of the anti-HN and anti-F responses. In analyzing the CTL response to Sendai virus, Sugamura et al. (14) used targets treated with UV-inactivated virus and UV-inactivated virus that had been treated with trypsin to inactivate the fusion function of F. The results of these experiments seemed to indicate that F was the functional target for the CTL response. As noted above, we documented the elution of HN-PCRV from the surface of cells within 2 h at 37°C and observed that incorporation of HN required active F in the same PCRV. It is possible, therefore, that an anti-HN response was not detected by Sugamura et al. because the virus had eluted from the surface of the target cell. Our present results indicated greater anti-F activity than anti-HN activity in the CTL response; however, response to both glycoproteins was clear. Hale et al. (7) explored the issue of major histocompatibility complex restriction and target antigen identity in experiments with liposome-borne H-2 glycoproteins and viral glycoproteins to modify target cells. These authors showed that only cells bearing syngeneic H-2 molecules and HN and F were lysed by anti-Sendai virus CTL. They pointed out that the attachment function of HN and the fusion function of F were both necessary to introduce liposome-borne glycoproteins into target cell surfaces. In this regard, their findings are in concordance with ours. They were unable to discriminate between anti-HN and anti-F activity because, as they pointed out, they could not introduce each glycoprotein independently.

ACKNOWLEDGMENTS

These investigations were supported by funds from the Saudi Arabian Educational Mission to the U.S. and a Research Competi-

tion Award from the School of Medicine, State University of New York at Buffalo.

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