tained 13 g of hydrolyzed starch per 100 ml of buffer. The gel buffer was a 1 : 10 dilution of the bridge buffer; the bridge buffer was 0.1*M* tris, 0.1*M* maleic acid and 0.01*M* $MgCl_2$ adjusted to *p*H 7.7 with NaOH. Hydrolyzed starch was purchased from Connaught Medical Research Co., Toronto, Canada. Chemicals and agar powder type IV were obtained from Sigma Chemical Co., St. Louis, Missouri. All enzymes were obtained from Boehringer, Mannheim, Germany.

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11. Under ether anesthesia, heart puncture was performed and blood was drawn into ethylenediaminetetraacetate (EDTA) solution. The elements of blood were separated according to R. Silber, B. W. Gabio, F. M. Huennekens [*J. Clin. Invest.* **41**, 230 (1962)]. Blood cells were lysed by freezing and thawing twice.
12. Linear sucrose gradients with 17 to 38 percent sucrose in 50 m*M* tris-HCl, *p*H 7.4, and 2 m*M* $MgSO_4$ were prepared with the Beckman density gradient former. The gradients were made in polyallomer tubes (1.2 by 7 cm); total volume was 4.8 ml. Centrifugation was carried out in the SW65 K rotor of the Spinco L2-65 centrifuge at 2°C for 18 hours at 55,000 rev/min. The bottoms of the tubes were punctured with a needle, and fractions of approximately 0.17 ml were collected.
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15. Hemoglobin was estimated by absorbance at 410 nm.
16. Supported by a PHS grant HD 2147-05. E.M. is a PHS Fogarty international fellow 1 FO5 TW 1635-01.

3 May 1971 ■

Human Lymphocyte Antigen Reactivity Modified by Neuraminidase

Abstract. *Human lymphocytes treated with neuraminidase (from* Vibrio cholera*) are more susceptible to lysis with antiserums directed against HL-A antigens in the cytotoxicity test than are the corresponding cells incubated in buffer. Enzyme-treated cells are also lysed by antibodies other than those directed against HL-A, but control cells are not. The extra sensitivity to antibody disappears after 2 to 6 days in tissue culture.*

Blood and tissue cells of many types and from diverse species show a change in antigenicity after treatment with neuraminidase (sialidase), an enzyme known to cleave the 2,3- and 2,6-glycosidic linkages between terminal sialic acid residues and cell surface mucopolysaccharides (*1*). Currie *et al.* (*2*) showed that A_2G mouse trophoblast cells, treated with neuraminidase and injected into CBA mice, would elicit second-set responses when the mice were tested 14 days later with A_2G skin grafts. Untreated trophoblasts did not elicit this response. Currie and Bagshawe (*3*) found that four out of five methylcholanthrene-induced mouse tumors failed to grow in compatible hosts after treatment with neuraminidase, although exposure to neuraminidase did not kill the cells. Sanford reported that 6 percent of C3H mice were susceptible to the allogeneic TA3 tumor after neuraminidase treatment, compared with a 56 percent susceptibility to this tumor in mice injected with untreated cells (*4*). Gasic and Gasic (*5*) used a subline of the TA3 tumor, which has a heavy sialomucin coat at the cell surface, to demonstrate the removal of the coating after neuraminidase treatment. Kraemer (*6*) reported regeneration of the sialomucin layer of a Chinese hamster cell line 16 to 20 hours after neuraminidase treatment. This regeneration could be inhibited by the addition of puromycin to the culture medium. Although in vitro methods have been used to study the removal and regeneration of sialomucin, antigen studies have been largely inferential since they have relied heavily on in vivo demonstrations and changes in reactivity. We now report our use of the two-stage cytotoxicity test (*7*) to determine the in vitro reactivity of neuraminidase-treated human lymphocytes both before and after they were placed in tissue culture.

Lymphocytes taken for culture purposes were prepared aseptically (*7*), with buffered calcium saline (CBS) (*8*) used as a final suspension medium in place of barbital buffer.

Vibrio cholera neuraminidase (Behringwerke or Calbiochem) was used at a concentration of 2 units per 5×10^6 cells suspended in 1 ml of CBS (*9*). An equivalent volume of neuraminidase-free CBS was added to the control cells and both sets were incubated at 37°C for 20 minutes. The cells were then washed once in one volume of CBS and resuspended in barbital buffer at the original concentration of 5×10^6 cell/ml.

Both neuraminidase and sham-treated cells were also washed once in RPMI 1640 (Grand Island) before being cultured at a concentration of 1×10^6 cell/ml in this medium to which the following were added: heat-inactivated human serum (10 percent), 1 ml of glutamine per 100 ml, 25 mg of streptomycin per 100 ml, and 50,000 units of penicillin G per 100 ml. The reactions of the cells in the two-stage cytotoxicity test (*7*) were examined on day 0 and at intervals of 1 to 2 days during culture. In the cytotoxicity test, 1 μl of serum and 1 μl of lymphocytes were incubated together in a microtiter plate (Falcon) at 25°C for 15 minutes. The cells were then washed with barbital buffer, and 1 μl of rabbit serum, which had been absorbed with 0.1 ml of packed human lymphocytes or spleen cells, was added as a source of complement. The plates were incubated at 37°C for 15 minutes, trypan blue–ethylenediaminetetraacetate was added, and the percentage of dead cells was determined microscopically.

Over a wide range the amount of neuraminidase and the time of incubation required to increase lymphocyte sensitivity are not critical. Cells were tested for sensitivity by the two-stage cytotoxicity test at several dilutions of antibody. To test for nonspecific cytotoxicity the same test procedure was used except the cells were incubated in barbital buffer instead of in antiserums. One unit of neuraminidase produced severe damage to 5×10^5 lymphocytes within 20 minutes, as was evidenced by an increase in the number of cells staining with trypan blue in control tests. Although as little as 0.02 unit incubated for 1 minute would increase sensitivity in some tests, 0.2 unit for 20 minutes gave high sensitivity with little or no loss of viability and was adopted for regular use. The supernatant obtained after exposing 5×10^5 lymphocytes to 0.2 unit of neuraminidase was still active while enzyme heated at 37°C for 3 hours or in a boiling water bath for 10 minutes failed to increase lymphocyte sensitivity.

The type of suspension medium and the absorption of the complement were important to the stability of the cells. Variability in trypan blue exclusion was found when lymphocytes suspended in saline, Hanks solution, or barbital buffer were treated with neuraminidase. Good viability was more consistently obtained when the cells were prepared in CBS. The volume of suspending medium was not critical over the range of 10 to 200 μl of CBS per 5×10^5 cells when 0.2 unit of enzyme were used. Absorption of the rabbit complement with human leukemic lymphocytes or spleen cells was essential to avoid damage from a heterophile antibody in nor-

mal rabbit serum. This antibody will kill 100 percent of enzyme-treated cells, reaching a titer of 1:4 to 1:8, but is readily removed by absorption.

Cells from 150 unrelated subjects and from members of 22 families have been tested against 11 serums that have never reacted in direct tests with untreated cells and against 88 antiserums directed against HL-A. Every serum showed an increased frequency of reactivity. This increase was specific. The HL-A haplotypes were detected in families by serums that failed to react with untreated cells. Patterns were sometimes given by several serums which did not coincide with the inheritance of HL-A and the reciprocal of these patterns could be discerned in certain families. The neuraminidase-dependent reactions were characteristic of the individual and were consistent. That the effect was not due to indiscriminate killing of the cells was shown by experiments in which enzyme-treated and sham-treated cells from 11 donors were placed in tissue culture. Figures 1 and 2 show the reactions obtained with cells from two subjects. Twelve antiserums reacted with saline-treated cells from donor RC (Fig. 1), as compared to 44 with enzyme-treated lymphocytes. After 4 days in culture, most of the extra reactivity was lost, and by day 7 the reactions were almost identical to those of the untreated original cells. Similar changes are apparent with cells from donor TCr (Fig. 2), but in two separate experiments with these cells regeneration appeared more quickly than in the other ten subjects tested. Very little cell proliferation was observed during any of the cultured cell experiments.

Treating human lymphocytes with neuraminidase increases the reactivity of the cells with serums in the two-stage cytotoxicity test. It is doubtful that the increased sensitivity of treated cells is caused by increased fragility. Enzyme-treated cells give excellent negative controls in the cytotoxicity test, will give negative cytotoxicity reactions with many serums, survive well in tissue culture for 6 to 8 days, and respond as well to PHA stimulation as control cells do. The regeneration time of sialic acid on human cells (in terms of a loss of enzyme-induced sensitivity in the cytotoxicity test) appears to be longer than the time reported for regeneration in mice (*5*) and hamsters (*6*). However, we have not investigated the connection between changes in the amount of sialic acid on the cell and the loss of cytotoxicity sensitivity. Sialic acid does not appear to be a part of the antigen, as no cytotoxicity reactions disappeared after enzyme treatment.

The removal of sialic acid by the enzyme may change the steric configuration or the zeta potential of the membrane to make antigens more accessible to antibodies, thus increasing the binding of complement. The CYNAP reaction (cytotoxicity negative-absorption positive serum-cell reaction) (*10*) is evidence that many HL-A antibodies can combine with cells in the presence of complement and not produce lysis. A CYNAP cell will not react with a serum in the two-stage cytotoxicity test, but will absorb reactivity for other cells from that serum. An example of such a reaction in our experiments is the heterophile antibody of normal rabbit serum which can easily be removed by absorption with untreated lymphocytes or spleen cells, but which does not give a cytotoxicity reaction with such cells. Since the fixation of complement by im-

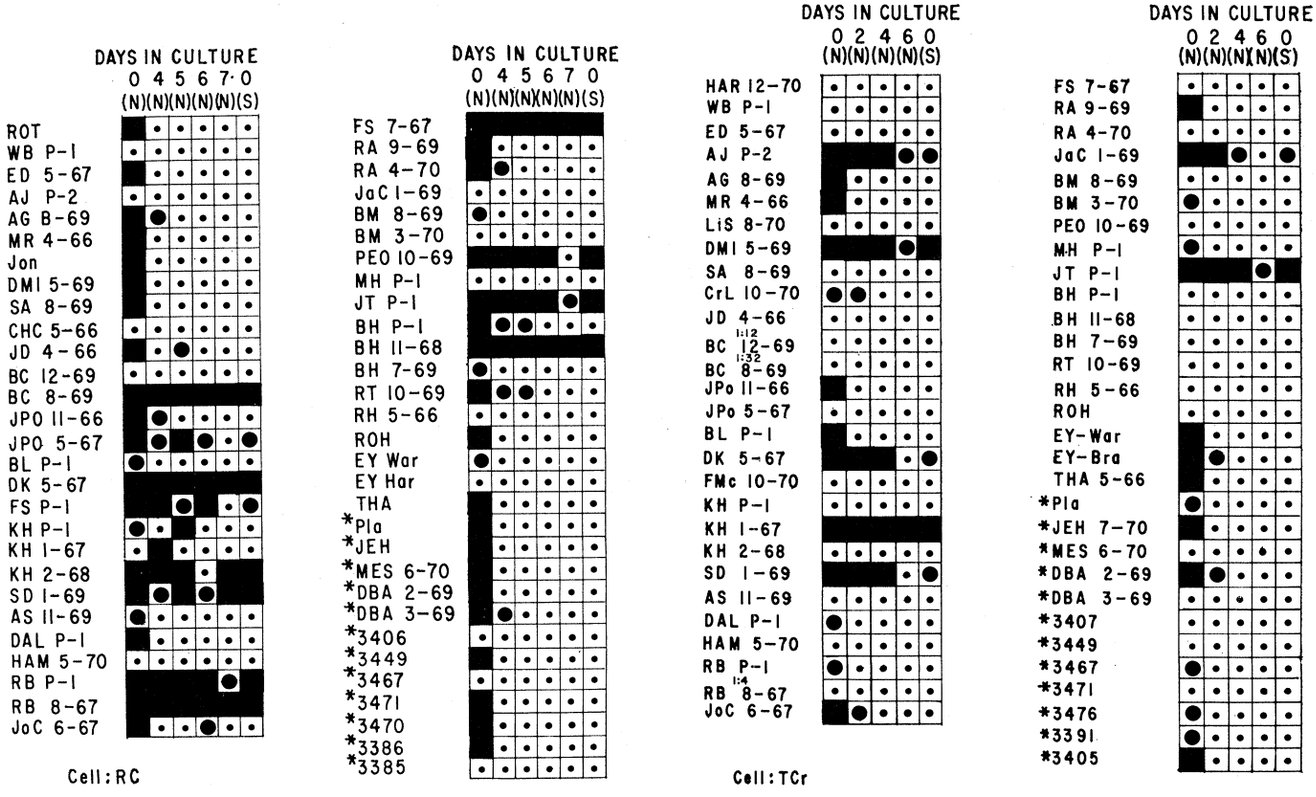

Fig. 1 (left). Reaction of cells from donor RC with serum panel before tissue culture [day 0 (N) and day 0 (S)] and on days 4, 5, 6, and 7 after culture. (N), Neuraminidase-treated cells; (S), sham-treated cells; ■, 60 to 100 percent lysis; ●, 20 to 59 percent lysis; •, 0 to 19 percent lysis. Fig. 2 (right). Reaction of cells from donor TCr with serum panel before tissue culture [day 0 (N) and day 0 (S)] and on days 2, 4, and 6 after culture. (N), Neuraminidase-treated cells; (S), sham-treated cells; ■, 60 to 100 percent lysis; ●, 20 to 59 percent lysis; •, 0 to 19 percent lysis.

munoglobulin G (IgG) is thought to depend on the simultaneous attachment of two IgG molecules (*11*), perhaps neuraminidase treatment allows more antibody to attach to the cell in such a manner that more complement can be bound. It is also possible, since Ray *et al.* (*12*) found that rabbit serum treated with cobra venom used as complement was fully lytic for neuraminidase-treated cells and that the removal of sialic acid uncovers a site essential for the completion of the lytic action of complement rather than the initiation step of complement fixation. Whatever the mechanism causing the increase in sensitivity of treated cells, it appears from our studies that many other CYNAP-like reactions are occurring for both HL-A and other lymphocyte antigens and that these reactions give positive cytotoxicity tests when neuraminidase-treated lymphocytes are used.

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8. The buffered calcium saline contained 0.145*M* NaCl, 0.003*M* $CaCl_2$, and 0.004*M* $NaHCO_3$ in H_2O.
9. One unit of enzyme activity is defined by Behring Diagnostics, Inc., as the amount of enzyme necessary to release 1 μg of *N*-acetylneuraminic acid from an acid α-1-glycoprotein substate in 15 minutes at 37°C and *p*H 5.5. The original sample of Behring neuraminidase used in the experiments (provided by Dr. B. Sanford) was reported free of detectable proteolytic activity by J. F. Codington [*J. Nat. Cancer Inst.* **45**, 673 (1970)].
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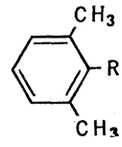
Oxidative N-Dealkylation: A Mannich Intermediate in the Formation of a New Metabolite of Lidocaine in Man

Abstract. *Evidence is presented for a new metabolite of lidocaine. Its structure,* N^1*-ethyl-2-methyl-*N^3*-(2,6-dimethylphenyl)-4-imidazolidinone, suggests reactive electrophilic intermediates for the oxidative removal of* N*-alkyl groups in general.*

The local anesthetic lidocaine **1** has been useful for the control of acute cardiac arrhythmias. However, despite its extensive clinical use, little is known about its metabolism in man.



1 R, $NHCOCH_2N(CH_2CH_3)_2$

2 R, $NHCOCH_2NHCH_2CH_3$

3 R, NH_2

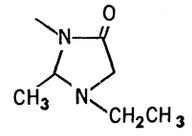
4 R, $NH-C\equiv\overset{+}{O}$

5 R, NH^+

6 R, +

7 R, $NHCOCH_2N$ 

8 R, $NHCOCH_2N(CH_2CH_3)(CH{=}CH_2)$

9 R, 

In 1966 Beckett *et al.* reported that after intravenous administration of **1** (three subjects), under conditions where the urine was maintained at constant acidic *p*H, approximately 10 percent of the administered dose could be accounted for as **1**; ω-ethylamino-2,6-dimethylacetanilide, **2**; and 2,6-dimethylaniline, **3** (*1*). To our knowledge these are the only metabolites of **1** that have been reported as occurring in man.

We now report our studies on this problem, in particular the finding of a new metabolite, and on the basis of the structure of this new metabolite we suggest the possibility of a reactive electrophilic intermediate for enzymatically mediated *N*-dealkylation reactions in general.

Three normal subjects were given 500 mg, by mouth, of the hydrochloride of **1**, containing 50 μc of randomly tritiated **1** as hydrochloride. Feces and urines of these subjects were collected, frozen, and stored over a period of 72 hours at which time 50 percent of the administered radioactivity had appeared in the urine. Since most of the radioactivity appeared in the urine within the first 8 hours after administration this fraction was selected for the initial isolation of metabolites.

A fraction composed of organic bases containing approximately 12 percent of the activity present in the urine was obtained by making the urine basic, exhaustively extracting it with ether, and the back extracting the ether fraction with aqueous acid. The ether was evaporated and the residue contained little activity, as did the residues after evaporation of ether extracts of urine obtained at various *p*H's. In addition, prior treatment of portions of the urine with a mixture of glucuronidase and sulfatase, followed by extraction with ether under a variety of conditions yielded insignificant activity in these organic extracts.

Since we expected to find **1** and **2** in the base-containing fraction, we sought conditions that would allow their separation and characterization by means of gas-liquid chromatography (GLC). Subsequently, a system (5 percent Carbowax on KOH-washed Chromosorb W, 1.8 m, 165°C) that gave excellent separation was found. In addition a high-resolution mass spectral study was made of **1** and **2** in order to elucidate the structures of any unknown metabolites. The study indicated that, as expected (*2*), both compounds fragmented primarily by homolytic cleavage of the carbon-carbon bond between the carbonyl and methylene groups to generate immonium ions at *m/e* 86 ($C_5H_{12}N$) and *m/e* 58 (C_3H_8N) as the base peak ions in the spectra of **1** and **2**, respectively. Exact measurements of the ions at *m/e* 148, 120, and 105 are consistent with structures **4**, **5**, and **6**, respectively. These structures represent