Vol. 96, No. 3, 1980
October 16, 1980

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 1184-1191

INHIBITION OF MYOBLAST FUSION BY TUNICAMYCIN AND PANTOMYCIN

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Received August 26,1980

Summary: The formation of myotubes by a continuous rat myoblast line, L6, can be inhibited by non-toxic concentrations of tunicamycin and pantomycin. The effect of tunicamycin, an inhibitor of UDP-N-acetylglucosamine: dolichol phosphate N-acetylglucosaminyltransferase, could be reversed by N-acetylglucosamine but not by mannose, glucose or UDP-N-acetylglucosamine.

Introduction: In the process of skeletal muscle differentiation (myogenesis), individual mononucleated muscle precursor cells or myoblasts initially proliferate and then fuse to form multinucleated bodies called myotubes. The sequential events in myotube formation in a permanent rat myoblast cell line, L6, isolated by Yaffe (1) are being studied in our laboratory (2). We have been interested in the role of the various cell surface glycoconjugates in the fusion process. It has been demonstrated that radioactive labelling of a ganglioside, GD1a, increases just prior to fusion in L6 (3) and a protein of apparent molecular weight of 70 kdaltons accumulates concomitant with the onset of fusion in chick myoblasts (4).

As an aid to the identification and study of the various macromolecules involved in fusion, we have investigated the effect of tunicamycin, an inhibitor of glycosylation (review, Ref. 5) and of several

Abbreviations: TMN, tunicamycin; PTN, pantomycin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Man, D-mannose; GlcN, D-glucosamine; GlcNAc, N-acetyl-D-glucosamine.

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other antibiotics on myoblast differentiation. We report that TMN and pantomycin inhibit the fusion of L6 myoblasts and that inhibition by TMN can be reversed by N-acetyl-D-glucosamine.

Materials and Methods

Materials: Tunicamycin was obtained through the kind services of C.G. Burrell of Eli Lilly and Co. (Canada) Ltd. Pantomycin was a gift of Dr. S. Gurusiddaiah, Washington State University. Amphomycin was a gift of W.F. Minor, Bristol Laboratories. Showdomycin, bacitracin, trypsin and 2-deoxy-D-glucose were from Sigma Chemical Co. Pronase, Hepes and UDP-N-acetyl-D-glucosamine were from Boehringer Mannheim Canada Ltd. The following radiochemicals were obtained from New England Nuclear: D-[U-^{14}C]-mannose (226.8mCi/mmol), D-[2-^{3}H]-mannose (18.4 Ci/mmol), D-[1-^{14}C]-glucosamine (54.2mCi/mmol) and [methyl-^{3}H]-thymidine (20.0 Ci/mmol). L-[4,5-^{3}H]-leucine (52 Ci/mmol) was from Amersham Corp. Bio-Gel P-10 (200-400 mesh) was from Bio-Rad Laboratories (Canada) Ltd.

Fusion Index and Growth Curves: L6-9/1, a clone of L6, and a non-fusing variant isolated from it were grown as described previously (6). Growth curves were obtained by plating 10^4 cells in a 6 cm tissue culture plate. The drugs when used were added in fresh media one day after plating. Cells were harvested by trypsinization and were counted by Coulter Counter. Fusion indices were determined ten days after plating and plating efficiences seven days after plating as described previously (6).

Cells were grown in minimal essential media (Gibco, U.S.A.) supplemented with 10% (v/v) fetal calf serum and 20 mM D-glucose. Pantomycin was dissolved in 95% (v/v) ethanol and tunicamycin in a solution containing equal parts of dimethyl sulfoxide and phosphate-buffered saline.

Incorporation of Radioactive Precursors: 10^4 cells were plated in each well of a 24-well tissue culture plate. The next day the medium was replaced by that containing TMN. After ten days the required tracer, D-[1-^{14}C]-Man, [methyl-^{3}H]-thymidine, or L-[4,5-^{3}H]-leucine each at a concentration of 1 µCi/ml was added. After 2 hr at 37°C, incorporation was terminated by removing the medium and washing successively three times with phosphate-buffered saline, once with 1% (w/v) phosphotungstic acid dissolved in 0.5 N HCl, and three times with 10% (w/v) trichloroacetic acid. The precipitate was dissolved in 1N NaOH for scintillation counting. Protein was determined by the method of Lowry et al (7).

Preparation of glycopeptides: 4 x 10^5 cells were plated in each of several 10 cm tissue culture plates. One day after plating, the medium was replaced by fresh medium containing TMN. After exposure to the drug for 24 hr, D-[2-^{3}H]-Man (5 µCi/ml) and D-[1-^{14}C]-GlcN (0.5 µ Ci/ml) were added to the plates. After another 24 hr, cells in each plate were washed three times with citrate-saline and then exposed to 1.5 ml trypsin (1 mg/ml) dissolved in 50 mM Hepes, pH 7.5, containing 100 mM NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$ and 0.04% (w/v) NaN_3. After 15 min at 37°C, the suspended cells were centrifuged and the supernatant was collected. It was supplemented with 2 mg/ml pronase and incubated for 24 hr at 37°C. At this time another 2 mg/ml pronase was added and the incubation continued for a further 24 hr period. The pronase digest was then boiled for a few minutes to destroy the pronase and centrifuged at 10,000 x g for 10 min to remove precipitated

material. The supernatant was applied to a Bio-Gel P-10 column (1 x 120 cm) and developed with 100 mM NH_4HCO_3. One ml fractions were collected and counted.

Results

Effect of TMN and PTN on growth and myoblast fusion: TMN at a concentration less than 0.35 µg/ml did not affect the growth rate of L6 or the plating efficiency of the cells (Fig. 1). Myoblast fusion, however, was inhibited even at concentrations as low as 0.15 µg/ml. This is evident from Fig. 1 where the plating efficiency curve at several TMN concentrations has been plotted along with the per cent fusion to emphasize that inhibition of fusion occurred at a TMN concen-

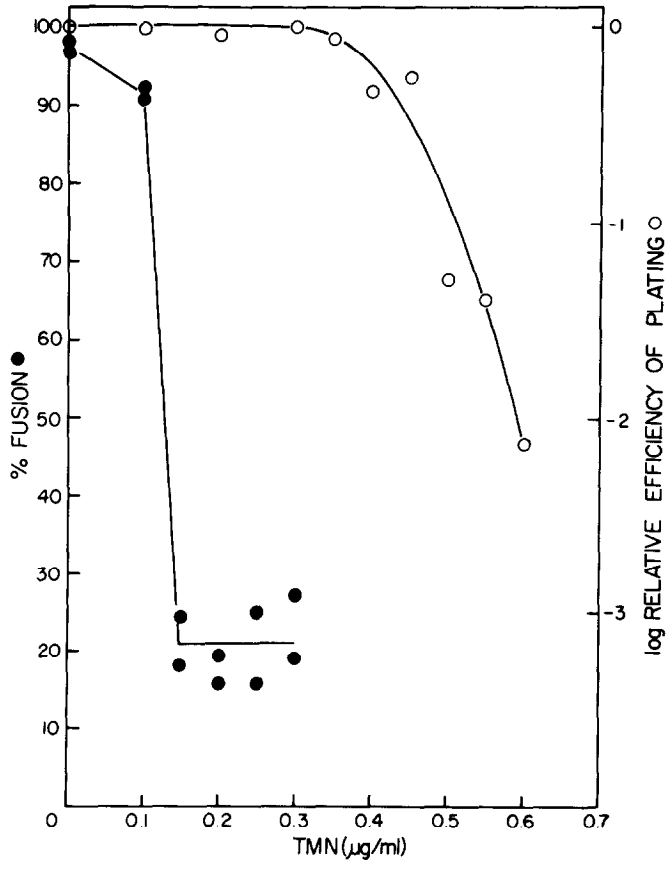

Fig. 1. Effect of TMN on % Fusion and Relative Efficiency of Plating of L6-9/1

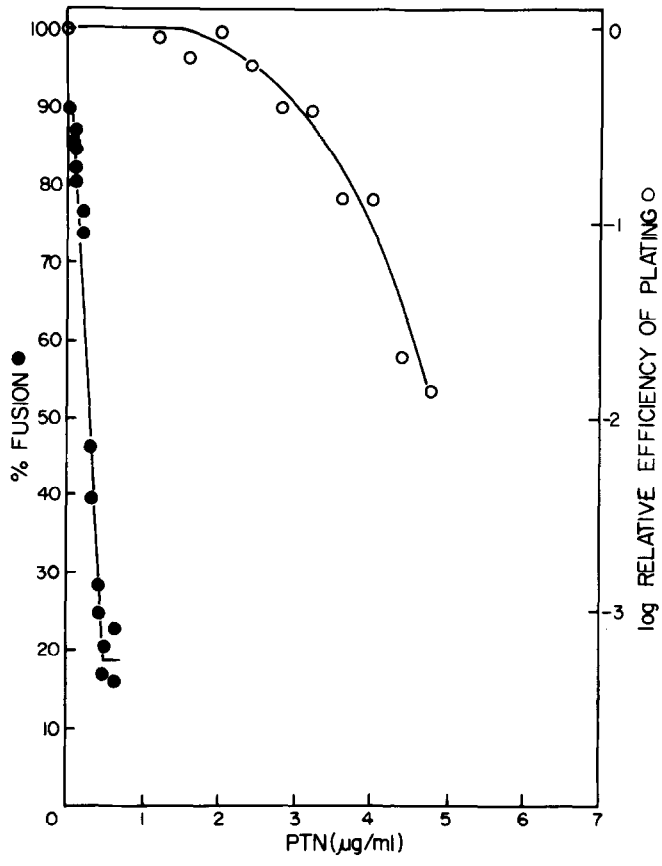

Fig. 2. Effect of PTN on % Fusion and Relative Efficiency of Plating of L6-9/1

tration less than one-half of that at which killing became obvious. Pantomycin also inhibited myoblast fusion at concentrations (0.05-0.6 µg/ml) well below that required to reduce plating efficiency (>1.2 µg/ml) to any significant extent (Fig. 2.).

TMN did not stimulate [^{3}H]-thymidine incorporation or induce thymidine kinase activity in a non-fusing variant of L6 isolated in our laboratories (data not shown).

The following compounds did not inhibit myoblast fusion: 0.8 mM 2-deoxy-D-glucose, 200 µg/ml amphomycin, 1 mg/ml bacitracin and 8 µg/ml showdomycin.

Table 1. Effect of TMN on Leucine, Thymidine and Mannose Incorporation Into Acid-Insoluble Material

TMN (µg/ml)	[^{3}H]-Leucine (pmol/mg)	%	[^{3}H]-Thymidine (nmol/mg)	%	[^{14}C]-Mannose (nmol/mg)	%	Relative Amount of Fusion %
0	759±91[a]	100	1.15±0.11[a]	100	12.0±0.1[a]	100	100
0.20	720	94.9	6.96	605	5.76	48.0	21±4[b]
0.25	921	121	8.89	773	5.83	48.6	13±0[b]
0.30	836	110	9.76	844	4.31	35.9	11±3[b]

[a] ±SD, n=3

[b] ±1/2 difference between duplicates

Effect of TMN on the Incorporation of Label Into Acid-Insoluble Material: Under conditions where TMN inhibited myoblast fusion, [^{3}H]-leucine and [^{3}H]-thymidine incorporation into acid-insoluble material were not inhibited (Table 1). In fact [^{3}H]-thymidine incorporation appeared to be stimulated several-fold. This was a reflection of the fact that myoblasts continued to synthesize DNA and divide in the presence of TMN, while control cultures lacking the drug ceased to produce DNA in preparation for fusion. In accord with this interpretation and in contrast to leucine and thymidine, [^{14}C]-mannose incorporation was inhibited over 50%. [^{3}H]-glucosamine incorporation, however, was not inhibited (results not shown).

Profiles of Radiolabelled glycopeptides: Glycopeptides were dual-labelled with D-[2-^{3}H]-Man and D-[1-^{14}C]-GlcN after exposing the cells to TMN for a short period. No differences were observed in the elution patterns of glycopeptides obtained from treated and control cells (Fig. 3a and 3b). In Fig. 3 peak 1 was identified as glycosaminoglycan, and peaks 2 and 3 as consisting of complex type glycopeptides and high mannose type glycopeptides, respectively (8).

Reversion of Fusion Inhibition by N-acetylglucosamine: Addition of GlcNAc reversed the inhibition of fusion caused by TMN in a dose-dependent manner (Table 2). Reversal was not obtained with 50 mM

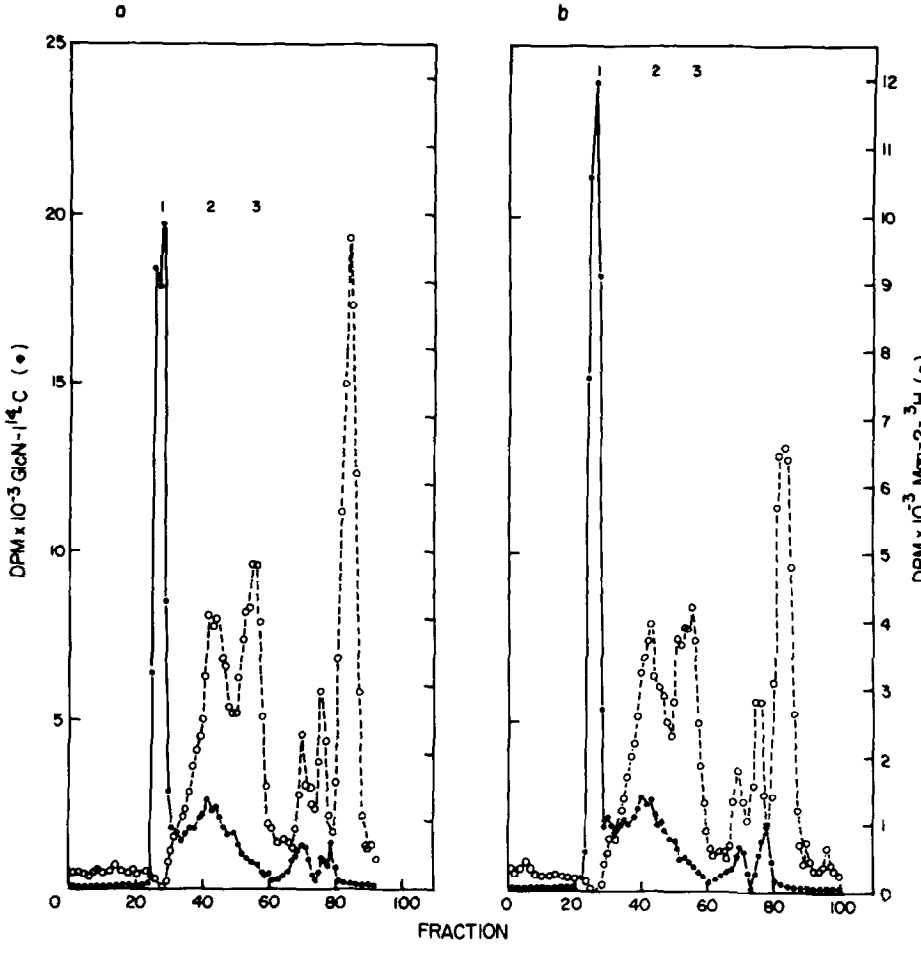

Fig. 3. Gel Filtration Profile of [^{3}H]-Man and [^{14}C]-GlcN-labelled Glycopeptides Prepared from L6-9/1
3a, control; 3b, 0.16 μg/ml TMN

Table 2. Effect of N-acetylglucosamine on the Inhibition of Myoblast Fusion by Tunicamycin[a]

N-acetylglucosamine (mM)	% Relative Degree Of Fusion[b]
0	25
5	30
10	45
25	65

[a] 0.2 μg/ml

[b] Control with no tunicamycin was defined as 100% Fusion. Each point was an average of duplicates.

Man, 100 mM D-glucose, 25 mM D-xylose, 1 mM UDP-N-acetyl-D-glucosamine or by growth on a gelatin substrate. Fusion inhibition produced by PTN could be reversed neither by GlcNAc nor by mannose.

Discussion

TMN inhibited myoblast fusion at non-toxic concentrations (Fig. 1). This inhibition was not simply due to the toxicity of the drug as judged by the observation that at fusion inhibiting concentrations of TMN no changes in plating efficiency or growth rates were observed. TMN did not inhibit [^{3}H]-Leu incorporation suggesting that overall protein synthesis was not greatly affected. TMN only inhibited [^{14}C]-Man incorporation and fusion suggesting that a fusion-specific glycoprotein(s) was not synthesized in the presence of TMN. These glycoproteins would have to be dispensible for growth, as fusion-inhibiting concentrations of TMN are without any perceptible effect on growth and viability. These possibilities are supported by the observation that the effect of TMN on fusion was reversed by GlcNAc (Table 2).

We have ascertained that TMN at a concentration of 0.2 µg/ml did not effect some other cellular processes in myoblasts which probably require involvement of glycosylated proteins. These were wheat germ agglutinin binding (9), production of cetylpyridinium Cl-insoluble material (10), production of collagenase-sensitive material (11), cell-cell adhesion (12) and transport of 2-deoxy-D-glucose and α-aminoisobutyric acid (13).

Pantomycin, a polypeptide ionophore for K^{+}, Rb^{+} and Li^{+} (S. Gurusiddaiah, personal communication), also inhibited myoblast fusion at non-toxic concentrations (Fig. 2). That it was not inhibiting glycosylation was suggested by the observations that neither Man nor GlcNAc reversed its effects and that a wheat germ agglutinin resistant mutant of L6, WGA^{RI}, isolated in our laboratory which lacks terminal sialic

acid and galactose residues and is cross-resistant to tunicamycin and showdomycin (14) was not cross-resistant to PTN.

These results suggest that some glycoproteins not essential for growth are necessary for myoblast fusion but their nature and exact role remains to be elucidated. As we were unable to observe any gross changes in cell surface glycopeptides (Fig. 3), one possible explanation for the inhibition of fusion by TMN is that one or more glycoproteins required for the fusion process while synthesized were underglycosylated. Such underglycosylated glycoproteins may be more sensitive to denaturation or just not incorporated into the cell membrane (15, 16).

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