- Enzymol. 303:179-205.
5. **Lander, E.S.** 1999. Array of hope. *Nat. Genet.* 21:3-4.
6. **Maskos U. and E.M. Southern.** 1992. Oligonucleotide hybridizations on glass supports: a novel linker for oligonucleotide synthesis and hybridization properties of oligonucleotides synthesised in situ. *Nucleic Acids Res.* 20:1679-1684.
7. **Molecular Probes SYBR Green II Product Information Sheet.**
8. **Pease, A.C., D. Solas, E.J. Sullivan, M.T. Cronin, C.P. Holmes and S.P. Fodor.** 1994. Light-generated oligonucleotide arrays for rapid DNA sequence analysis. *Proc. Natl. Acad. Sci. USA* 91:5022-5026.
9. **Pietu, G., R. Mariage-Samson, N.A. Fayein, C. Matingou, E. Eveno, R. Houlgatte, C. Decraene, Y. Vandenbrouck et al.** 1999. The Genexpress IMAGE knowledge base of the human brain transcriptome: a prototype integrated resource for functional and computational genomics. *Genome Res.* 9:195-209.
10. **Ramsay, G.** 1998. DNA chips: state-of-the art. *Nat. Biotechnol.* 16:40-44.
11. **Schena, M., D. Shalon, R.W. Davis and P.O. Brown.** 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270:467-470.
12. **Schena, M., D. Shalon, R. Heller, A. Chai, P.O. Brown and R.W. Davis.** 1996. Parallel human genome analysis-microarray-based expression monitoring of 1000 genes. *Proc. Natl. Acad. Sci. USA* 93:10614-10619.

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Received 20 December 1999; accepted 16 March 2000.

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Transmembrane Motility Assay of Transiently Transfected Cells by Fluorescent Cell Counting and Luciferase Measurement

BioTechniques 29:81-86 (July 2000)

ABSTRACT

Current in vitro assays used in assessing tumor motility could be improved by the development of a simple technique that would facilitate studies of the impact of specific genes on pharmacologically altered chemotaxis. We developed a technique that improves on the classic transwell assay by using fluorescence and luminescence to assess chemotaxis. In this transient transfection system, co-transfection of a reporter construct and a gene with an unknown impact on motility are coupled with biochemical assays to quantitate the number of cells that have received a transferred gene, which subsequently crosses the membrane. This assay was found to be less variable than the conventional transwell chamber and is easily adaptable to studies of cell motility or cell invasion. We also demonstrate that this assay can detect the effect of both genetic and pharmacological inhibition of motility alone and in combination. It therefore has the potential to reveal additive or synergistic effects.

INTRODUCTION

A tumor's transition from a hyperproliferative to an invasive lesion is associated with a significant decrease in patient survival. A window of opportunity thus exists before the invasive disease develops. This has resulted in significant efforts to develop novel therapies that delay or prevent such an invasion. Directed tumor cell motility or chemotaxis is the final step of tumor invasion, and the inhibition of this process has been a major focus of research. Several modifications of the transwell assay have been used to quantitate both the invasive and chemotactic response of cells. In the classic assay,

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staining and counting of the cells that have moved through the membrane are carried out (1). A few modifications to this classic assay have been made, for example, the use of computer image analysis programs to count the cells (9) or assess their movement (2); the use of a densitometer to estimate the number of cells (10); and staining cells with toluene blue to visually inspect and count them, then solubilizing the dye and quantitating the absorbance of the blue dye at 600 nm (11). Another method is to prelabel the cells with radioactivity (13) or fluorochromes and then quantitate the amount of label passing through the membrane (15).

Still another method is based on the MTT cleavage assay (14) that depends on endogenous enzymatic activity. This method is vulnerable to artifacts when the tested compound or gene impacts the metabolic machinery of the cell without necessarily affecting tumor motility. Currently, studying the role of a gene in chemotaxis can be done either by generating stably transfected cell lines for use in transwell assays or by single-cell motility analysis following transient transfection using video time-lapse microscopy (4). However, these approaches are not amenable to large drug-screening efforts.

Our goal was to improve the transwell chamber design (8) that has been used successfully to measure both invasion (1) and motility (5). By using quantitative biochemical methods for endpoint assessment, we reduced the experimental variability of the procedure. In combination with cells transiently transfected with reporter constructs, we demonstrated that this assay allows rapid identification of the roles new genes play in chemotaxis and also determines the efficacy of anti-chemotactic compounds.

MATERIALS AND METHODS

Cell Lines, Tissue Culture and Inhibitors

The T24 cell line was established from an invasive human bladder cancer and obtained from ATCC (Manassas, VA, USA). The cell line contains a mutated (val12) *ras* gene and is highly

motile in vitro (18). RT10 (16) and T24T (6) human bladder cell lines were derived in our laboratory and also express a mutated (val12) *ras* gene (12). All cell lines were maintained in DMEM:F12 (1:1) media (Life Technologies, Rockville, MD, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS). Serum-free medium (SFM) consisted of HyClone® CCM™ medium (HyClone Laboratories, Logan, UT, USA). The cells were harvested using 0.25% trypsin and resuspended in PBS in preparation for passaging. Counting cells in suspension was carried out using a model ZM Coulter Counter™ (Coulter, Miami, FL, USA). Wortmannin (Sigma, St. Louis, MO, USA) (19), a selective irreversible inhibitor of phosphatidylinositol 3-kinase (PI3K), was applied to cell cultures in the concentrations described in the figure legends.

DNA Constructs and Transfection

See the earlier description of the human *ras* dominant negative (p21-RasN17) construct (7). The pRL-CMV vector containing the strong constitutive cytomegalovirus (CMV) immediate-early enhancer/promoter driving the Renilla luciferase gene was purchased from Promega (Madison, WI, USA). The pEGFP-N1, a construct expressing enhanced green fluorescent protein (GFP), was purchased from Clontech Laboratories (Palo Alto, CA, USA). Five micrograms of plasmid DNA prepared by the standard cesium chloride gradient method were used for transfecting 5×10^6 cells by electroporation using a Gene Pulser™ II (Bio-Rad Laboratories, Hercules, CA, USA). For cotransfections, we used a total of 5 µg DNA in a ratio of 2:1 of p21RasN17 DNA to pRL-CMV DNA. Following transfection, the cells were plated and incubated for 24 h before assay.

Chemotaxis Assay

Estimation of cells plated on polyethylene terephthalate (PET) filters. To obtain a quantitative assessment of the cells that were plated on the superior surface of the PET filters, we simultaneously plated an equal number of cells in separate 24-well plates. Following a

similar period of incubation, the cells were harvested from these plates and overall cell numbers estimated using the CyQUANT® assay (Molecular Probes, Eugene, OR, USA) and transfected cell numbers using the Dual Luciferase assay® (Promega). The Falcon™ Track etched PET membrane (Becton Dickinson, Franklin Lakes, NJ, USA) we used was commercially treated in a way similar to tissue culture plates and were expected to have similar adhesive properties for the cells used. We verified this by plating similar numbers of cells in both the 24-well plate and on the superior surface of the transwell chambers. Following a 6-h incubation without chemoattractants, the cell numbers in both were estimated using the CyQUANT assay and Coulter counter.

Classic and modified transwell assay. For the assay, blind well transwell chemotaxis chambers with 6.5-mm diameter, 8-µm thick, polyvinylpyrrolidone-free PET filters with 8-µm pore size (Becton Dickinson) were used. H1 medium containing 5% heat-inactivated FBS was placed in the lower compartment of the well and used as a source of chemoattractants. There was little cell migration (2%–7%) in the absence of FBS in the lower compartment over a 6-h period (data not shown) and no passive diffusion because the pores of the filter were smaller than the cells. Cells suspended in H1 medium were added to the upper chamber and assays carried out as described earlier (17,18). For our modified assay, the same filters, media, chemoattractants and incubation conditions as already described for the classic transwell assay were used. At the end of the incubation period, the cells on the upper surface of the filter were completely removed by wiping with a cotton swab. The filters were then processed as described for the CyQUANT assay and the Renilla luciferase assays. The motile fraction is defined as the CyQUANT or luciferase relative light units (RLU) of cells on the inferior surface of the membrane, divided by the CyQUANT or luciferase RLU of cells on the replica plate. The use of CyQUANT or luciferase RLU depended on whether we were measuring the migration of the total cell population across the filter or just that of transfected cells.

Renilla Luciferase and CyQUANT Assays

An estimation of transiently transfected cells plated on the superior surface of PET filters was performed by lysing cells in the 24-well replica plates as described earlier, according to the Dual Luciferase assay kit instruction manual (Promega). To estimate the number of transfected cells on the inferior surface of the PET filter, filters were placed in a 1.5-mL Eppendorf[®] tube (Hamburg, Germany) before processing in a way similar to the cells in replica plates described earlier. Luciferase activity, expressed as RLU, was evaluated on a TD-20/20 Lumino-meter[™] (Turner Designs, Sunnyvale, CA, USA). The CyQUANT assay for cell-number quantitation is based on the use of a proprietary green fluorescent CyQUANT GR dye, which exhibits strong fluorescence enhancement when it is bound to cellular nucleic acids. An aliquot of the cell lysate was used for fluorescence measurement on a Biolumin[®] 960 kinetic fluorescence/absorbance microplate reader (Molecular Dynamics, Sunnyvale, CA, USA).

RESULTS

The CyQUANT assay was linear and sensitive within the ranges tested and was not affected by Matrigel[™]. The fluorescence that was emitted by the CyQUANT GR dye at 520-nm increases in proportion to both the cell count and lambda DNA concentration and is linear from 50 or fewer cells to at least 50 000 cells in 200-μL volumes. Increasing the dye concentration extends the linear range to 250 000 cells. Thus, the sensitivity of the assay was about 50 cells, or 50 pg/mL DNA. When the cells were counted using a Coulter counter, the relative fluorescence units were consistent from run to run [for 30 000 cells measured by the Coulter counter: 42 866 RLU ± 1333 SD; for 3000 cells: 6741 RLU ± 452 SD]. Cell counts in the replica wells, as assessed by the CyQUANT and Coulter counter, were similar to those on the superior surface of the transwell assay when no chemoattractants were used. This indicates the replica plates were a

true reflection of the cells present in the superior surface of the transwell.

To determine if this assay could be extended to the study of tumor-cell invasion using Matrigel-coated transwell chambers (1), we explored whether or not this product could induce artifacts of either the CyQUANT or luciferase assays. Following a 24-h recovery period, cells transfected with pRL-CMV were plated in transwell inserts and tissue culture plates. After a 6-h incubation, the samples in the transwell inserts and tissue culture plate were assessed for CyQUANT and luciferase with and without Matrigel added before lysis. No difference in the readings of either CyQUANT or luciferase was noted as a function of the presence or absence of Matrigel coating (data not shown).

The range of 25 000 to 30 000 cells placed in the upper chamber was optimal for assessment of cell migration. Because we were looking for the inhibitory action of genes and drugs on motility, we tested a range of cell numbers placed in the upper chamber and the replica plate. We hoped to determine the functional range of cell numbers that would produce both a robust signal and a lack of significant inhibition of motility from crowding on the upper surface of the filter. At 50 000 cells, we found an appreciable inhibition of motility, probably because of competition for the holes. There is no statistically significant difference in the motility index at cell numbers less than 50 000. Hence, 30 000 cells were used in the standard assay for assessing the effects of pharmacologic agents on motility. Although the assay is capable of detecting as few as 50 cells, we found that conditions resulting in greater than 300 cells on the underside of the filters were more reproducible (data not shown). We also assessed the sensitivity of the modified assay using a GFP construct (pEGFP-N1) rather than pRL-CMV, but this reporter was found to be significantly less sensitive than the latter (data not shown). Finally, we wiped both sides of six filters following incubation with 30 000 cells for 6 h and determined that 2%–3% of the CyQUANT GR dye fluorescence signal remains on the filter, indicating that a negligible number of cells remain trapped on the filter (data not shown).

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The modified assay is more reproducible and more sensitive than the classic transwell assay. We compared the reproducibility of the modified and classic transwell assays by measuring the migration of T24 cells. The intra-assay coefficients of variation were $\pm 5\%$ and $\pm 17\%$, respectively, for the modified and classic assays. The interassay coefficients of variation were $\pm 7\%$ and $\pm 15\%$ respectively. We also evaluated the two assay methods at decreasing initial cell counts. When 30 000 cells were plated in the upper chamber, the intra-assay coefficients of variation were $\pm 3\%$ for the modified assay and $\pm 14\%$ for the classic assay. With lower numbers of cells plated on the top surface of the filter, the intra-assay coefficient of variation was less than $\pm 25\%$ for as few as 4000 cells, while the classic assay required more than 16 000 cells per well to achieve the same reproducibility.

The luciferase assay is an accurate and sensitive means of measuring the motility of transfected cells. Before demonstrating that the modified assay can detect the effect of different gene constructs on cell motility in a transient co-transfection assay, we sought to determine if the reporter construct itself has adverse effects on this assay. As

seen in Figure 1, the motile fraction of 30 000 T24 cells transfected with the pRL-CMV reporter construct is similar, whether or not it was measured by luminescence (only cells that have taken up pRL-CMV were measured) or CyQUANT (all cells were measured) ratios. In addition, the effect that wortmannin has on the motility of T24 is similar in both the luminescence assay and the CyQUANT assay. These results indicate that the pRL-CMV reporter does not alter the motility of these cells.

The effect of gene constructs on motility can be determined in a simple transient transfection assay. Here, we demonstrate that the modified assay can allow effective evaluation of the impact of a gene on tumor cell motility and invasion. This was done by demonstrating the importance of p21RasV12 in conferring the motile (17) and invasive (16) phenotype to bladder cancer cells. We transfected the dominant negative p21RasN17 gene into T24, T24T or RT10 cell lines and examined its effect on motility using the modified assay. Figure 2 confirms that the modified assay demonstrates the expected result of interruption of the *ras* signaling pathway, namely a decrease in T24 cell motility. This was also true for T24T

and RT10 (data not shown).

These data also reveal a statistically significant difference between the CyQUANT and Luciferase assays when p21RasN17 was transfected ($P < 0.05$). This difference in the observed inhibition of cell motility between the two assays was because the CyQUANT assay measures all cells (i.e., transfected and nontransfected) while the luciferase assay measures only transfected cells. Thus, despite our high transfection efficiency (about 50%), the motile fraction measured by CyQUANT would be expected to be higher (i.e., fewer cells inhibited) than that measured by Luciferase. Finally, detecting simultaneous inhibition of motility by genetic and pharmacologic agents was investigated by treatment of p21RasN17 transfected cells with wortmannin. We have previously shown that wortmannin inhibits the motility of T24 cells (18) and expected that this would be reproduced using the modified assay. As seen in Figure 2, the combination of wortmannin and dominant negative *ras* inhibits cell motility to a greater extent than p21RasN17 alone. This demonstrates that our modified assay can simultaneously reveal the effects of both genetic and pharmacological inhibition of motility.

Figure 1. Chemotaxis assay of T24 in serum-free medium with and without addition of wortmannin: comparison of the CyQUANT assay to the Renilla Luciferase assay. 30 000 T24 cells, transfected with luciferase reporter construct pRL-CMV 24 h earlier, were resuspended in H1 medium with and without 10 μ M wortmannin and placed in the upper compartment. Equal numbers of cells with and without wortmannin were replica-plated in standard 24-well tissue culture plates. At the end of a 6-h incubation period, the modified assay was carried out as described in Materials and Methods. The motile fraction represents the ratio of fluorescence or luminescence RLU of the cells on the undersurface of the PET filter to that of the replica wells. Means $\pm$ SD ($n = 4$) are shown.

Figure 2. Chemotaxis assay of p21RasN17 transfected T24 cells in serum-free medium with and without the addition of wortmannin. 30 000 T24 cells transfected with luciferase reporter construct pRL-CMV (Control), alone or together with the p21RasN17 (N17) expression construct 24 h earlier (see Materials and Methods). They were then harvested from tissue culture plates, suspended in H1 medium with and without 10 μ M wortmannin (wort) and placed in the upper compartment. H1 media containing 5% FBS was placed in the lower compartment to serve as a chemoattractant. Equal numbers of cells with and without wortmannin were replica-plated in standard 24-well tissue culture plates. At the end of the 6-h incubation period, the modified assay was carried out as described in Materials and Methods. The motile fraction was calculated as in Figure 1. Means $\pm$ SD ($n = 4$) are shown.

DISCUSSION

A large percentage of the interassay variation observed with the classical transwell assays came from the uneven distribution of cells on the lower membrane and the difficulty in counting clumped cells (9). While counting all the cells on the lower portion of the membrane addressed the former issue, the latter issue was much more difficult to solve. However, counting all the cells is currently impractical and fields are chosen at random. The cell counts in each are averaged together to yield a final result. Our assay overcame this problem by effectively quantifying all the cells on the undersurface of the membrane while simultaneously and significantly reducing the processing time and variability of the assay. In addition, by quantitating cells using assays that measure nucleic acids instead of protein, our assay can reliably assess

nuclear translocation, a critical component of cell motility. This reduction in variability has the beneficial effect of allowing more subtle differences in motility to be documented when comparing various experimental conditions. Finally, because of the sensitivity of the assay, this approach may allow the design and construction of miniaturized assay reagents, which, in turn, may lead to the eventual high-throughput screening of agents for their effects on cell motility.

Most current variations of the transwell assay assume (but do not evaluate directly) that an equal number of cells are capable of attaching to the upper portion of the membrane in each experimental condition. Thus, a construct containing a gene that negatively affects the plating efficiency of cells could potentially lead to completely erroneous conclusions with respect to its effect on motility. Unless the gene transfer is accomplished in situ, that is, on cells al-

ready on the upper surface of the membrane, then a separate plating efficiency control is needed, as shown in our assay.

The human transitional cell carcinoma cell lines, T24, T24T and RT10, overexpress the p21RasV12 oncogene. Since we previously determined that activated p21RasV12 leads to increased chemotaxis (17), we reasoned that overexpression of a dominant negative mutant of *ras* (p21RasN17) in these cells should inhibit their chemotactic response. This experiment would then demonstrate that our assay reveals the effects of transiently transfected genes on cell motility. Not unexpectedly, the addition of this construct strongly inhibited the motility of these cells in response to chemotactic factors and so validated our system as useful in the genetic study of motility.

Because our assay is based on cotransfection of a reporter construct and an experimental gene, another practical

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concern is the effect of the gene of interest on the reporter. A recent study showed that several genes have effects on the promoter reporter systems used to tag cells (3). Because our system assays both the upper and lower sides of the membrane, motility should not be affected. This conclusion is suggested by our p21RasN17 experiment. Here, the RLU of the upper and lower chambers in p21RasN17-transfected cells were lower than in the control, and the gene should have had the same effect on the cells on both sides of the membrane. Its effect on the promoter system of the luciferase gene should not have impacted conclusions reached regarding cell motility.

In view of the growing interest and potential promise of chemogenetic therapy for cancer, we demonstrated how the assay could be used to study the simultaneous activity of genes and drugs on cell motility. Since we previously showed that T24 motility is inhibited by the phosphatidylinositol 3-kinase inhibitor wortmannin (18), we used this

agent with p21RasN17 to study the combined effects of these agents on T24 motility. This reports shows that wortmannin inhibits the chemotactic response of T24 cells, an effect that is still evident in the context of *ras* inhibition. Finally, even though we have demonstrated our assay with motility as an end point, the system can be adapted easily to the study of tumor invasion using Matrigel coating (1) of PET filters since this product does not seem to interfere with the fluorescent readouts.

REFERENCES

1. Albini, A., Y. Iwamoto, H.K. Kleinman, G.R. Martin, S.A. Aaronson, J.M. Kozlowski and R.N. McEwan. 1987. A rapid in vitro assay for quantitating the invasive potential of tumor cells. *Cancer Res.* 47:3239-3245.
2. al Moustafa, A.E., N. Urbani and M. O'Connor-McCourt. 1999. Black cellular spreading and motility assay. *BioTechniques* 27:60-62.
3. Behre, G., L.T. Smith and D.G. Tenen. 1999. Use of a promoterless Renilla luciferase vector as an internal control plasmid for transient co-transfection assays of Ras-mediated transcription activation. *BioTechniques* 26:24-27.
4. Chicoine, M.R. and D.L. Silbergeld. 1997. Mitogens as motogens. *J. Neurooncol.* 35:249-257.
5. Fong, C.J., D.M. Sutkowski, J.M. Kozlowski and C. Lee. 1992. Utilization of the Boyden chamber to further characterize in vitro migration and invasion of benign and malignant human prostatic epithelial cells. *Invasion Metastasis* 12:264-274.
6. Gildea, J.J., W.L. Golden, M.A. Harding and D. Theodorescu. 2000. Genetic and phenotypic changes associated with the acquisition of tumorigenicity in human bladder cancer. *Genes Chromosomes Cancer* 27:252-263.
7. Hartsough, M.T., R.S. Frey, P.A. Zipfel, A. Buard, S.J. Cook, F. McCormick and K.M. Mulder. 1996. Altered transforming growth factor signaling in epithelial cells when ras activation is blocked. *J. Biol. Chem.* 271:22368-22375.
8. McCarthy, J.B. and L.T. Furcht. 1984. Laminin and fibronectin promote the haptotactic migration of B16 mouse melanoma cells in vitro. *J. Cell Biol.* 98:1474-1480.
9. Minkin, C., D.J. Bannon, Jr., S. Pokress and M. Melnick. 1985. Multiwell chamber chemotaxis assays: improved experimental design and data analysis. *J. Immunol. Methods* 78:307-321.
10. Mrowietz, U. and G. Jurgens. 1995. A method for the determination of leukocyte migration for large sample numbers by automated densitometric quantification. *J. Biochem. Biophys. Methods* 30:49-58.
11. Muir, D., L. Sukhu, J. Johnson, M.A. Lahorra and B.L. Maria. 1993. Quantitative methods for scoring cell migration and invasion in filter-based assays. *Anal. Biochem.* 215:104-109.
12. Parada, L.F., C.J. Tabin, C. Shih and R.A. Weinberg. 1982. Human EJ bladder carcinoma oncogene is homologue of Harvey sarcoma virus ras gene. *Nature* 297:474-478.
13. Repesh, L.A. 1989. A new in vitro assay for quantitating tumor cell invasion. *Invasion Metastasis* 9:192-208.
14. Schlechte, W., M. Brattain and D. Boyd. 1990. Invasion of extracellular matrix by cultured colon cancer cells: dependence on urokinase receptor display. *Cancer Commun.* 2:173-179.
15. Schratzberger, P., C.M. Kahler and C.J. Wiedermann. 1996. Use of fluorochromes in the determination of chemotaxis and haptotaxis of granulocytes by micropore filter assays. *Ann. Hematol.* 72:23-27.
16. Theodorescu, D., I. Cornil, B.J. Fernandez and R.S. Kerbel. 1990. Overexpression of normal and mutated forms of HRAS induces orthotopic bladder invasion in a human transitional cell carcinoma. *Proc. Natl. Acad. Sci. USA* 87:9047-9051.
17. Theodorescu, D., K.R. Laderoute, J.M. Calaoagan and K.M. Guldin. 1998. Inhibition of human bladder cancer cell motility by genistein is dependent on epidermal growth factor receptor but not P21ras gene expression. *Int. J. Cancer* 78:775-782.
18. Theodorescu, D., K.R. Laderoute and K.M. Guldin. 1998. Epidermal growth factor receptor-regulated human bladder cancer motility is in part a phosphatidylinositol 3-kinase-mediated process. *Cell Growth Differ.* 9:919-928.
19. Ui, M., T. Okada, K. Hazeki and O. Hazeki. 1995. Wortmannin as a unique probe for an intracellular signalling protein, phosphoinositide 3-kinase. *Trends Biochem. Sci.* 20:303-307.

This work was supported in part by National Institutes of Health grant no. CA75115-01, a scholarship from the Kimmel foundation for D.T. and NIH training grants for J.J.G. and M.A.H. Address correspondence to Dr. Dan Theodorescu, Box 422, University of Virginia Health Sciences Center, Charlottesville, VA 22908, USA. e-mail: theodorescu@virginia.edu

Received 10 November 1999; accepted 15 March 2000.

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