Direct Antilymphoma Effects on Human Lymphoma Cells of Monotherapy and Combination Therapy with CD20 and HLA-DR Antibodies and ^{90}Y-Labeled HLA-DR Antibodies

Gerald L. DeNardo, Evan Tobin, Kaylund Chan, Bonnie M. Bradt, and Sally J. DeNardo

Abstract **Purpose:** Monoclonal antibodies (mAb) in combination and mAbs combined with a radionuclide (radioimmunotherapy) have both been more effective in patients than mAb monotherapy.

Experimental Design: Using assays of cell growth and viability, the dose response and temporal characteristics of CD20 (rituximab) and HLA-DR (Lym-1) mAbs, singly and in combination, and of ^{90}Y-conjugated Lym-1 mAb have been characterized in five human lymphoma cell lines (B35M, Raji, SU-DHL-4, SU-DHL-6, and Ramos) spanning Burkitt's to diffuse large cell lymphoma. Although Ramos had a lower HLA-DR density, these cell lines were otherwise selected because of high cell surface CD20 and HLA-DR abundance. Assays of cell growth and death were done using microscopy and trypan blue dye.

Results: Lym-1 and rituximab, used singly, showed direct antilymphoma effects; those of Lym-1 were often more potent than those of rituximab. Combinations of these mAbs were more effective, sometimes synergistic, than either mAb singly, even in more resistant SU-DHL-4 cells. Conjugation of ^{90}Y to Lym-1 also augmented potency in all cell lines and overcame resistance to both Lym-1 and rituximab in Ramos cells.

Conclusions: Lym-1 exhibited substantially greater direct antilymphoma effects than rituximab in lymphoma cells in culture. Combination of Lym-1 with rituximab or ^{90}Y increased potency and overcame treatment resistance in lymphoma cells. Greater use of combination therapies of this type to increase potency and range of effectiveness seems likely to improve patient outcome.

Many antilymphoma monoclonal antibodies (mAb) have been shown to have biological activity *in vitro*. Several have also been shown to be effective for immunotherapy and radioimmunotherapy in patients as single agents. The chimeric CD20 mAb, rituximab, induces responses in ~50% of patients with low-grade follicular lymphoma although even these patients may relapse and become resistant to rituximab monotherapy (1, 2). In patients with aggressive lymphoma, rituximab has a lower response rate. Combinations of other mAbs with rituximab have been shown to be effective in patients (3, 4). Among reasons for resistance to one mAb and responsiveness to combinations of mAbs for immunotherapy can be the antigen density. Additionally, combinations of CD20 mAb with a radionuclide, for example ^{90}Y in the case of Zevalin (^{90}Y-labeled ibritumomab tiuxetan, Biogen Idec, Inc., Cambridge, MA) and ^{131}I in the case of Bexxar (^{131}I-labeled tositumomab, Corixa Pharmaceuticals, Seattle, Washington), have proven more effective for therapy than the mAb alone (2, 5). This has also been shown to be the case for the HLA-DR mAb Lym-1 (6). CD20 and HLA-DR antigens are expressed on many B-cell lymphomas at high surface densities and not modulated following mAb binding. Further, there is evidence of physical as well as functional interactions between these antigens that are members of the superantigen family (7–9). Finally, low-dose rate radiation, as from radionuclides, seems to operate through apoptotic mechanisms in common with mAb effects.

We have shown that the HLA-DR mAb, Lym-1, was more potent than rituximab in some cell lines and complemented the potency of rituximab (10). These *in vitro* observations are supported by those made in patients for the combination of rituximab and Hu1D10, another mAb against the HLA-DR β subunit (3).

The purpose of this publication is to report the results for combinations of Lym-1 and rituximab or Lym-1 and ^{90}Y on cell growth and viability of human lymphoma cell lines spanning Burkitt's and diffuse large lymphoma cells, and including cell lines with high CD20 and HLA-DR antigen density and one cell line having low HLA-DR antigen density (Ramos). The latter cell line was resistant to therapy with either and both mAbs.

Materials and Methods

Experimental design. Using assays for cell growth inhibition and viability, the dose response and temporal characteristics of the

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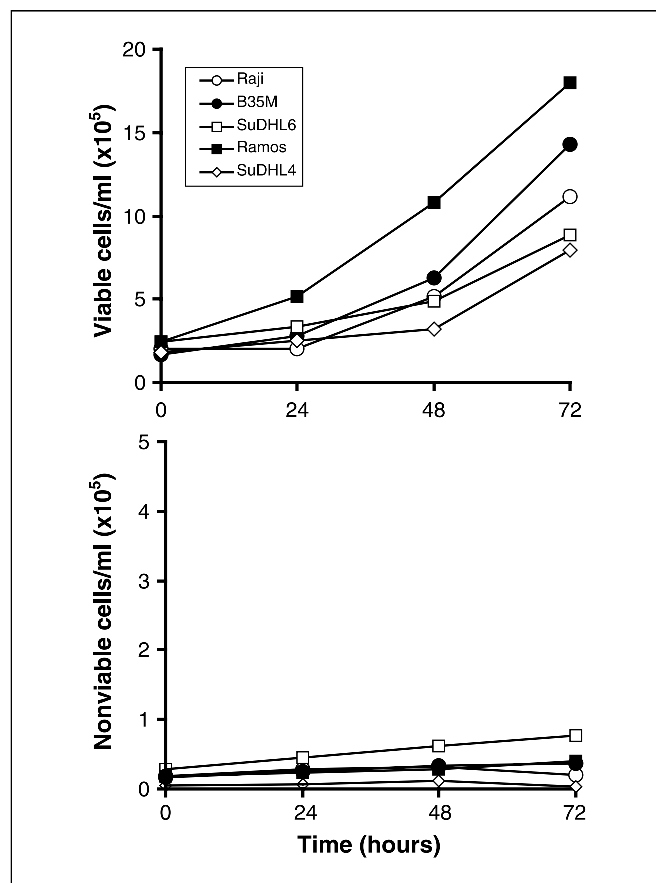

Fig. 1. Viable (*top*) and nonviable (*bottom*) cell counts over time. While growing in log phase, lymphoma cells were subcultured into wells; nonviable cells were initially less than 10% of total cells for all cell lines and assays. When untreated, viable cells continued to multiply logarithmically over the course of the assays; dead cells as a fraction of the total cells did not increase during the course of the assays. Untreated cells and cells treated with nonspecific mAbs were indistinguishable in their growth and death characteristics ($P > 0.16$).

antilymphoma effects of two mAbs were assayed in five human lymphoma cell lines. At mAb and ^{90}Y concentrations readily achieved in patients, the antilymphoma effects of rituximab, a CD20 mAb, and Lym-1, an HLA-DR mAb, were determined for each mAb and in combination with each other, or when ^{90}Y was conjugated to Lym-1. When the antilymphoma effects of ^{90}Y-Lym-1 were compared with those of Lym-1, mAb concentrations at time 0 were 10 μg/mL, a mAb concentration beyond which no additional antilymphoma activity was observed. To restrict effects, in other assays, to those for cell bound Lym-1 and ^{90}Y-Lym-1, media was replaced in all wells after 15 minutes. Lymphoma cell lines were chosen because they expressed the chosen antigens abundantly on their cell surfaces; Ramos, however, was chosen to determine the effect of antigen density as this cell line expresses CD20 abundantly but HLA-DR antigen expression is limited. Untreated samples and isotype-matched nonspecific mAbs were used as negative controls in each assay. Assays were replicated and replicates were included in individual assays to ensure reproducibility.

Antibodies. Murine mAb Lym-1 (Oncolym, Peregrine Pharmaceuticals, Tustin, CA) and chimeric Lym-1, kindly provided by Alan Epstein (University of Southern California, Los Angeles, CA), are B-lymphocyte specific and have high affinity for a discontinuous epitope on the β-chain of membrane-associated HLA-DR10 antigen (11). Lym-1 antigen is not shed from the lymphoma cell surface or internalized and it is up-regulated on malignant B cells (12).

Rituximab (Rituxan®, Genentech, San Francisco, CA; Biogen Idec, San Diego, CA), a chimeric immunoglobulin G1 mAb, binds to the CD20 antigen found on the surface membrane of mature and precursor B cells and also is not modulated.

Mouse and chimeric L6 (Oncogen, Seattle, WA) recognize a 202-amino-acid membrane-associated polypeptide, which exists on the surface of many types of epithelial cancer cells (13). mAbs L6 are not reactive with the HLA-DR10 antigen or any of the lymphoma cell lines used,[1] and served as isotype-matched control mAbs in the assays, herein described. Levels of endotoxin by the *Limulus* Amebocyte Lysate assay (BioWhittaker Pyrogent Plus, Walkersville, MD) were negligible in all mAb samples used in assay systems.

Radiopharmaceutical. Carrier free ^{90}Y (Perkin-Elmer, Boston, MA) was purchased as a chloride in dilute HCl. 7,10-Tetra-azacyclododecane-*N,N′,N″,N*-tetraacetic acid (DOTA)-peptide-Lym-1 was radiolabeled, as previously described (14) at specific activities for different products of 9.8 to 14.4 μCi/μg. Cellulose acetate electrophoresis and high-performance liquid choromatography revealed that ^{90}Y-DOTA-peptide-Lym-1 was >95% radiochemically pure with <5% aggregate content. A solid-phase immunoreactivity assay using Raji cells showed 60% to 76% binding of the ^{90}Y-DOTA-peptide-Lym-1 relative to an ^{125}I-Lym-1 reference standard (14, 15).

Cell lines. Raji and Ramos cell lines (human Burkitt's lymphoma) were purchased from the American Type Culture Collection (Rockville, Maryland) and maintained in RPMI 1640 growth medium with 10% FCS, 1% L-glutamine (200 mmol/L), 1% sodium pyruvate (100 mmol/L), 1% nonessential amino acids, and 1% penicillin/streptomycin at 37°C in a 5% CO_2 atmosphere. Human B-lymphoma cell lines B35M (Burkitt's) and SU-DHL4 and SU-DHL6 (diffuse large cell) were a gift from Alan Epstein and were maintained as above.

Assessment of cell number and viability. Microscopy and trypan blue dye exclusion assays were used to determine cell number and viability over 72 hours. Raji, B35M, SU-DHL-4, and Ramos cells were at least 95% viable and SU-DHL-6 cells at least 90% viable at initiation of assays. Cells (5×10^4), mAb, and/or medium (total volume, 200 μL) was added to 360 μL wells and then incubated at 37°C in a humidified 5% CO_2 environment. At each allotted time, replicate wells were harvested after pipetting to disrupt cell aggregates. Filtered trypan blue dye was added to the cells, and then cell number and viability were determined using light microscopy.

Calculation of radiation dose. Radiation doses (cGy) delivered to the lymphoma cells were calculated using medical internal radiation dose (MIRD) formalism (16), adjusted for volumes (masses) of distribution (17). β absorbed fraction for specific masses (volumes) were obtained by logarithmic interpolation of data from Siegel and Stabin (18), using a β energy of 0.937 meV for ^{90}Y and assuming uniform distribution in the mass.

Statistical analysis. To assess the significance of the differences in viable cell counts, a two-sample, one-tailed Student's *t* test was done on the replicate counts within an experiment. Results were deemed significant if $P \leq 0.05$.

Results

All five human lymphoma cell lines showed logarithmic growth of viable cells over 72 hours; nonviable (dead) cells were initially <10% of the total cells and did not increase fractionally over 72 hours (Fig. 1). These characteristic patterns of growth and death of the untreated cells were not altered by introduction of nonspecific mAbs into the media. When incubated with Lym-1, substantial growth inhibition and cell death occurred in B35M and Raji cells, and to a lesser extent with rituximab (Fig. 2). SU-DHL-6 cells were more sensitive to rituximab (data

[1] G. DeNardo, unpublished data.

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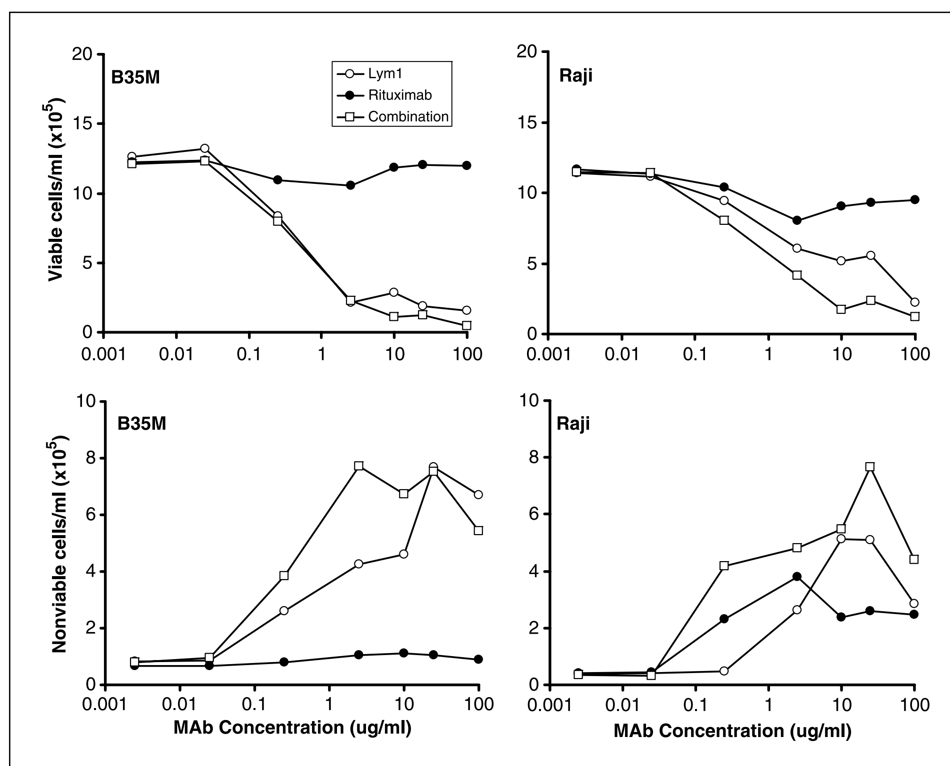

Fig. 2. Viable (*top*) and nonviable (*bottom*) cell counts, at 72 hours, of samples treated with rituximab, Lym-1, or combinations of these mAbs (concentrations on the abscissa are those of each mAb). Although rituximab and Lym-1 inhibited growth and induced death of B35M (*left*) and Raji (*right*) lymphoma cells, Lym-1 was more potent than rituximab in these cells ($P \leq 0.03$ at 10 μg/mL). Combination of Lym-1 and rituximab was more potent in B35M and Raji cells than either of the mAbs singly ($P \leq 0.04$ and ≤ 0.01 for Lym-1 or rituximab, respectively, at 10 μg/mL).

not shown). When incubated with a combination of rituximab and Lym-1, antilymphoma activity in B35M, Raji, and SU-DHL-4 cells was increased (Figs. 2 and 3). In other assays, the combination of rituximab and Lym-1 induced greater effects in B35M and SU-DHL-4 cells even when total mAb concentrations were equal (data not shown). The threshold concentrations for antilymphoma effects of the combination of Lym-1 and rituximab were lower than the thresholds for either Lym-1 or rituximab singly. Ramos cells were resistant to the combination or either mAb singly at all concentrations and times (Fig. 4).

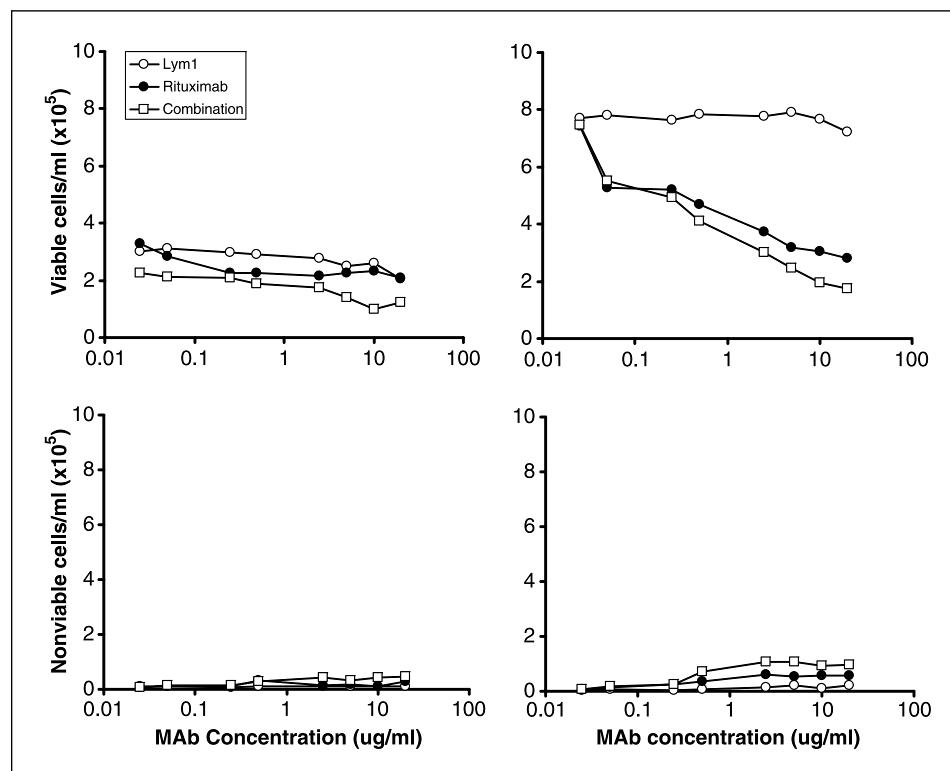

Fig. 3. Viable (*top*) and nonviable (*bottom*) SU-DHL-4 cell counts at 48 (*left*) and 72 hours (*right*). Over the first 48 hours, Lym-1 and rituximab showed little antilymphoma activity and the combination somewhat greater activity (concentrations on the abscissa are those of each mAb). By 72 hours, rituximab had induced antilymphoma effects that were increased by combination with Lym-1 ($P = 0.03$ at 10 μg/mL).

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However, ^{90}Y-conjugated Lym-1 increased growth inhibition in Ramos and all cell lines tested at about 500 cGy and more so at 5,000 cGy. These differences in antilymphoma effects occurred despite removal of unbound Lym-1 and ^{90}Y-Lym-1.

Discussion

Lym-1, whether mouse or chimeric, and rituximab had potent direct antilymphoma effects on human cell lines, spanning from Burkitt's to diffuse large lymphoma cells. Samples treated with Lym-1 or rituximab had fewer viable cells and more nonviable (dead) cells when compared with controls. In Raji and B35M cells, Lym-1 had greater effects than rituximab that were observed to be more prolonged in other studies (10). Lymphoma cells, when treated with nonspecific, isotype-matched mAbs, multiplied and remained viable, as if untreated. Although both rituximab and Lym-1 decreased the number of viable cells and increased the number of nonviable cells when added singly, their effects in combination were consistently greater in all cell lines tested, except Ramos cells. The difference between mono and combination mAb treatment seemed to be greatest in cell lines that exhibited some resistance to either rituximab or Lym-1 alone, as in B35M and SU-DHL-4 cells. When compared with either mAb alone, a substantial increase in dead B35M and Raji cells due to the mAb combination was observed. Even when adjusted for the total concentration of mAb, the antilymphoma effects of combination immunotherapy were disproportionately increased; greater antilymphoma activity was observed when giving a combination of these two mAbs than an equivalent dose of either mAb.

Clinical trials of combination immunotherapy, using rituximab with Hu1D10 (anti–HLA-DR; ref. 4), epratuzumab (anti-CD22; ref. 19), or alemtuzumab (anti-CD52; refs. 20, 21), have been conducted in patients with lymphoid malignancies. Preliminary data suggested a significant increase in response without increase in toxicity. Combining rituximab with Hu1D10, another HLA-DR mAb, has shown enhanced activity in patients (4). In the study reported here, we selected the combination of rituximab, a CD20 mAb, and Lym-1, an HLA-DR mAb, because the CD20 and HLA-DR antigens are expressed on a variety of B-cell lymphomas at high surface densities (12, 22). Further, there is evidence of physical and functional interactions between these molecules (7–9). HLA-DR mediates significant tyrosine phosphorylation and CD20 is a critical component in coupling MHC class II in B cells to phospho-tyrosine kinase signaling pathways (7). In one study, the effects of Lym-1 and rituximab, or combinations thereof, occurred in the absence of effector cells and complement, indicating that the effects occurred by direct mechanisms. Procaspase has been shown to be converted to caspase-3, and poly(ADP-ribose) polymerase induced, when lymphoma cells were treated with Lym-1 or rituximab or combinations thereof (10). Additive and synergistic effects between the two mAbs could be related to the linkage of CD20 and HLA-DR antigens on B cells.

Rituximab induces a therapeutic response in ~50% of patients with indolent lymphomas and has been rapidly incorporated into treatment regimens since approval (1). Nonetheless, patients become resistant to rituximab immunotherapy (23, 24). A potent way to augment the cytoreductive power of a mAb is to conjugate a radionuclide to the mAb. In the randomized, pivotal phase III trial of ^{90}Y-labeled ibritumomab tiuxetan, partial and complete remissions were several times greater in the ^{90}Y-labeled ibritumomab tiuxetan arm than in the rituximab arm (2). In our study, Ramos and SU-DHL-4 were rather resistant to Lym-1 and rituximab and combined treatment. The addition of low-dose rate radiation from ^{90}Y-Lym-1 bound to the lymphoma cells overcame resistance whether from limited antigen expression (Ramos cells) or Bcl-2 overexpression (SU-DHL-4 cells).

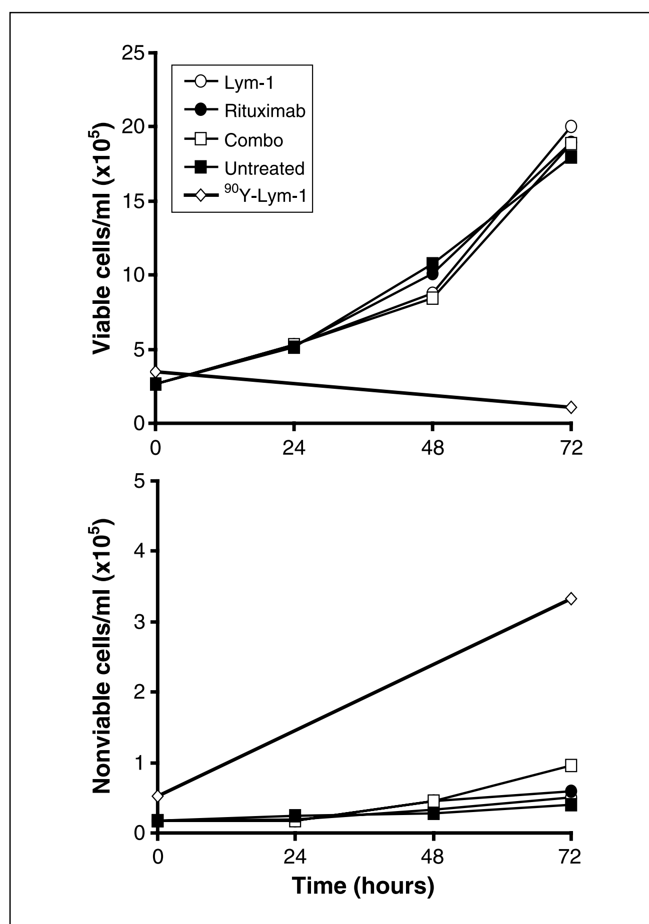

Fig. 4. Viable (*top*) and nonviable (*bottom*) cell counts over time. Ramos lymphoma cells were resistant to Lym-1 or rituximab singly and in combination, at all mAb concentrations and sample times. However, Ramos cells showed antilymphoma effects presumed due to the radiation from ^{90}Y-Lym-1 ($P < 0.001$ for ^{90}Y-Lym-1 versus Lym-1 at 72 hours). To ensure that these effects were due to specific binding of ^{90}Y-Lym-1 to the Ramos cells, media (unbound ^{90}Y-Lym-1 and Lym-1) was removed in other assays and replaced with fresh media at 15 minutes. These assays showed similar differences between ^{90}Y-Lym-1 – treated and Lym-1 – treated or untreated Ramos cells (data not shown). All mAb concentrations at time 0 were at 10 μg/mL, a concentration beyond which no additional antilymphoma activity was observed.

The results observed in this study show the advantages of combination therapy over monotherapy. The ability of the combinations to induce greater effects in various lymphoma cell lines, even those resistant to individual mAbs, suggests that combination therapy may be more effective in patients over a broader range of lymphoma types.

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