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Natural Cytotoxicity of NC-2$^+$ Cells Against the Growth and Metastasis of WEHI-164 Fibrosarcoma

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Abstract

We have previously reported a new receptor (NC-2) for natural cytotoxicity (NC) on murine leucocytes, identified by monoclonal antibody D9 (mAb D9). Pretreatment of mouse spleen cells with different concentrations of mAb D9 *in vitro* blocked NC against WEHI-164, whereas natural killing (NK) activity against YAC-1 was unaffected. This paper reports the immune surveillance against the growth of WEHI-164 tumour cells in mice by NC-2$^+$ Cells. The kinetics of *in vivo* reduction in NC activity were investigated by treating BALB/*c* and (CBA × C57BL/6) F1 mice with a single injection of 40 μg of mAb D9 and monitoring splenic NC activity by ^{51}Cr-release assay at intervals from 24 h to 3 weeks. Control mice were injected with OKT8 irrelevant antibody. Results showed a significant ($P < 0.05$) reduction in splenic NC activity within 24 h which persisted for up to 1 week. Similar results were also obtained when (CBA × C57BL/6) F1 mice were employed ($P < 0.001$). *In vivo* tumour studies were undertaken to investigate the role of NC-2$^+$ cells in surveillance against tumour growth and metastasis of the WEHI-164 fibrosarcoma. When syngeneic BALB/*c* mice were injected with 40 μg of mAb D9 and then challenged with 5×10^5 WEHI-164 cells, results showed significantly increased growth rate of the transplanted WEHI-164 fibrosarcoma and tumour nodules in the lungs of animals, when compared to control mice with normal NC activity. Our data support an innate surveillance in metastasis and growth of WEHI-164 fibrosarcoma in mice.

Introduction

Natural cell-mediated cytotoxicity is a major innate cellular defence mechanism against infection and cancer, and cell mediating natural killing (NK) and natural cytotoxicity (NC) are crucial elements of it [1]. The major differences between NK and NC are the receptors and mechanism involved in cytotoxicity [2–4]. The initial step in the mechanism of NK and NC is recognition and binding of effector cells to tumour cells via receptor–ligand interactions [5]. Cell-mediating NK were traditionally defined as cells that lyse lymphoid targets such as YAC-1 in short-term *in vitro* cytotoxicity assays [6], whilst cells-mediating NC were first characterized as cells that lyses non-lymphoid fibrosarcomas in long-term cytotoxicity assays [7]. NK-cell responses are mediated through cell-surface receptors that can either be inhibitory or activating.

An anti-tumour surveillance role for NC was originally proposed on the basis that spleen cells could mediate *in vitro* lytic activity against 3-MCA-induced fibrosarcomas [1, 5]. NK cells express a variety of activating and inhibitory receptors that use opposing signalling motifs to stimulate or inhibit activation [4]. These receptors induce signals, which the balance of these signals forms the decision of whether an NK cell becomes activated or activation is inhibited. This recognition system has some degree of flexibility, which means that NK cells use several strategies that allow their receptors to recognize diverse ligands. Although unlike T and B cells, flexibility is not achieved through the rearrangement of gene clusters, but NK-cell recognition receptor families have achieved flexibility through rapid genetic evolution (within a species) and reported promiscuity of ligand binding [4, 8, 9].

Cytotoxicity receptors (NCRs) are a group of activating receptors, which belong to the Ig superfamily [8]. The appearance of these receptors specifically NKp44 illustrated the rapid nature of the evolution of NK-cell receptors [10]. In humans, NCRs NKp46, NKp80 and

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NKp30 are expressed on activated and resting NK cells, but NKp44 is upregulated following interleukin-2 stimulation of some NK cells [11, 12]. Cellular ligands may exist, given that anti-NCR antibodies abrogate NK-cell-mediated lysis of many tumour cell types [13–17].

The NCRs are receptors triggering NK cells upon ligation to tumour targets [13, 16]. This is supported by studies showing that deletion of a single NCR reduces the ability of NK cells to lyse tumour targets *in vivo* [16, 18, 19]. These NCRs receptors are important in the use of NK cells in cancer therapy.

To our knowledge, only two receptors, NC-1.1 [20] and NC-2 [21], have been identified on murine leucocytes, which mediate NC against WEHI-164 (NC-sensitive target) whilst the activity of these cells against NK-sensitive target (YAC-1 tumour cells) was not affected. Studies showed that mice rendered deficient in NC by the administration of the anti-NC-1.1 mAb 1C4 have accelerated growth of a number of transplantable tumours [22] and exhibited increased susceptibility to chemical carcinogens [23] compared to control mice. These results provide the first direct evidence that in mice at least, NC has a role in tumour surveillance.

We have previously reported the production and characterization of an anti-NC mAb D9 that identifies the NC-2 receptor on cells mediating NC. NC-2 is a different receptor to NC-1.1. *In vitro* blocking studies showed that pretreatment of (CBA × C57BL/6) F1 mouse spleen cells with mAb D9 blocked NC against WEHI-164, whereas NK activity against YAC-1 (NK target) was unaffected. Furthermore, we have shown that mAbD9 blocked effector cells-WEHI-164 tumour cell conjugation approximately 50%, whilst conjugation to YAC-1 tumour cells was not affected [21]. We predicted from the *in vitro* data that mAb D9, like mAb 1C4, would reduce splenic NC when administered into mice. Therefore, an NC-deficient mouse model was generated to study the NC-2 tumour surveillance *in vivo*.

This paper reports the ability of mAb D9 (anti-NC-2) to deplete splenic NC *in vivo* and the use of these NC-deficient mice to study the growth and metastasis of the 3-MCA-induced WEHI-164 fibrosarcoma.

Materials and methods

Tissue culture medium (TCM). Dulbecco's modification of Eagle's medium (CSL, Melbourne, Vic., Australia) was supplemented with 20 mM 4-(2-hydroxyethyl)-1 piperazineethanesulfonic acid, 2 mM L-glutamine, 50 μM 2-mercaptoethanol 0.15% sodium bicarbonate, 50 μg/ml gentamicin and 10% foetal calf serum.

Animals. BALB/*c* and (CBA × C57BL/6) F1 mice of both sexes were selected according to our previous finding about the cytotoxicity of splenic leucocytes from different inbred mouse strains. The above-mentioned mice had higher NC activity against WEHI-164 tumour cells [21]. The animals were maintained in David Maddison Clinical Sciences Building Animal Facility under standard conditions and used between 10 and 12 weeks of age.

Cytotoxicity assay. Splenic NC activity was assayed by *in vitro* lysis of ^{51}Cr-labelled WEHI-164, as previously described [21]. The experiment was conducted in quadruplicate in 200 μl TCM in 96-well microtiter trays. Briefly, spleen cells were incubated with ^{51}Cr-labelled targets at effector/target (E:T) ratios in the range of 100:1–12.5:1. The cells were pelleted at 150 *g* for 1 min and incubated at 37°C/5% CO_2 for 18 h. Supernatants (100 μl) from each well were harvested and the level of radioactivity measured in a COBRA gamma-counter (Packard Instrument CO., Downer's Grove, IL, USA). The results were first calculated as percentage specific lysis:

$$\text{Percentage specific lysis} = \frac{\text{Test c.p.m} - \text{Background c.p.m}}{\text{Total release c.p.m} - \text{Background c.p.m}} \times 100$$

Background count per million (c.p.m) was obtained from wells containing target cells alone and total release was determined by counting ^{51}Cr-labelled target cells.

Time– and dose–response studies. To determine the dose–response effect of mAb D9 on BALB/*c* splenic NC activity, mice were injected intraperitoneally (i.p.) with 5, 10, 20, 40, 80 or 160 μg of mAb D9. Control mice were injected with equivalent doses of OKT8 (IgG2a), an isotype matched mAb. Twenty-four hours later, splenic NC activity of each mouse was assayed *in vitro* against ^{51}Cr-labelled WEHI-164 target. (CBA × C57BL/6) F1 mice were similarly tested for the effect of mAb D9 treatment *in vivo*. The minimal dose of mAb D9 required to achieve maximal reduction in splenic NC activity was 40 μg for both strains of mice; therefore, this dose was used throughout the study.

A time–response study for mAb D9 was carried out in BALB/*c* mice (n = 20) that were injected i.p. with 40 μg of mAb D9. At different times (24 h, 72 h, 1 week, 2 weeks and 3 weeks) after injection, five mice from each group were killed and their spleens were individually tested in ^{51}Cr-release assays against WEHI-164 targets. A similar experiment was carried out in (CBA × C57BL/6) F1 mice.

In vivo tumour growth studies. The MTD100 dose of WEHI-164 fibrosarcoma (a 3-methylclontren-induced BALB/*c* fibrosarcoma) was determined by subcutaneously injecting 5×10^5, 1×10^6 or 5×10^6 tumour cells into the chest of three groups (n = 10) of BALB/*c* mice. The animals were monitored for tumour growth every 2 days for up to 10 days after tumour cell inoculation. Results showed that all mice given a dose of 5×10^5 WEHI-164 cells grew tumours within 4 days, and this was the MTD100 dose used in subsequent experiment. To test the

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effects of mAb D9 on WEHI-164 tumour growth *in vivo*, two groups (n = 25) of BALB/*c* mice were used. Group 1 mice were i.p. injected with 40-μg affinity-purified mAb D9 in 200 μl of TCM. Group 2 mice were injected with a similar dose of OKT8 isotype-matched antibody. Monoclonal antibody treatments were carried out on days −2, 0, 2, 7 and 14 and 5×10^5 WEHI-164 cells were inoculated on day 0. The mice were monitored every 2 days and the tumours were measured as two diameters at right angles. The results are presented as mean tumour area (mm^2) ±SE according to the following formula [24].

$$\text{Mean tumour area} = \pi \times \left(\frac{\text{Diameter 1} + \text{Diameter 2}}{4}\right)^2$$

Tumour metastasis studies. The ability of WEHI-164 tumour cells to metastasis to the lungs was examined in this study. The minimal dose of cells to give 10–20 lung nodules in BALB/*c* mice was determined by intravenously (iv) injecting graded numbers (5×10^3, 1×10^4, 1×10^5 or 5×10^5) of WEHI-164 cells into the tail veins of groups (n = 8) of anaesthetized [15 mg/kg Rompun (Bayer, Sydney, Australia) and 100 mg/kg Ketamine (Parnell Laboratories, Alexandria, Australia) per mouse] BALB/*c* mice. The mice were killed on days 7, 10, 12 and 14 and the lungs were carefully removed, given a gentle rinse in saline and fixed in Bouin's solution (75 ml picric acid, 25 ml 40% formaldehyde and 5 ml glacial acetic acid). The number of lung tumour nodules was counted using a dissecting microscope. The regimen selected to study the effects of mAb D9 on tumour metastasis was i.v. injection of 1×10^5 WEHI-164 cells per mouse and harvesting the lungs 2 weeks later.

In this experiment, three groups (10 mice in each group) of mice were injected i.p. with 200 μl PBS containing 40 μg of mAb D9 or PBS alone as control, on days −2, 0, 2 and 7. On day 0, 1×10^5 WEHI-164 tumour cells were injected into the tail veins of anaesthetized mice. Two weeks after tumour cell inoculation, the mice were sacrificed and their lungs were removed and fixed in Bouin's solution. In this experiment, the tumour nodules in the lungs of the antibody-treated mice were too extensive to be counted, and hence, the lungs were weighed and the mean lung weights were compared amongst the three groups of mice.

Statistical analysis. The obtained data were reported as mean ± SEM. Student's t test or Mann–Whitney U test was used to determine statistical significance between test and control groups. A P-value of <0.05 was considered significant.

Results

The kinetics of *in vivo* reduction in NC activity was investigated by treating BALB/*c* and (CBA × C57BL/6) F1 mice. The animals were injected (i.p.) 40 μg of mAb D9. At different times (24 h, 72 h, 1 week, 2 weeks and 3 weeks) after mAb injection, five mice of each group were killed and their spleen cells were individually tested in ^{51}Cr release assay against WEHI-164 targets. Control mice were injected with OKT8 irrelevant antibody. Results for BALB/*c* mice are shown in (Fig. 1). A significant ($P < 0.05$) reduction in splenic NC activity was evident within 24 h which persisted for up to 1 week. The experiment was repeated in (CBA × C57BL/6) F1 mice with similar results (Fig. 2). The reduction in NC activity at 24 h was highly significant ($P < 0.001$) compared to control mice.

Role of NC-2$^+$ cells on the local growth of the WEHI-164 fibrosarcoma in BALB/*c* mice

Results of the experiment that examined the effects of a reduction in NC activity by mAb D9 (anti–NC-2) on the growth of the WEHI-164 fibrosarcoma are shown in Fig. 3. Between days 6 and 10 when the tumour area was <85 mm^2, mAb D9-treated mice consistently showed significantly increased growth of the tumour compared to control mice treated with the isotype-matched antibody ($P < 0.05$, Mann–Whitney U test).

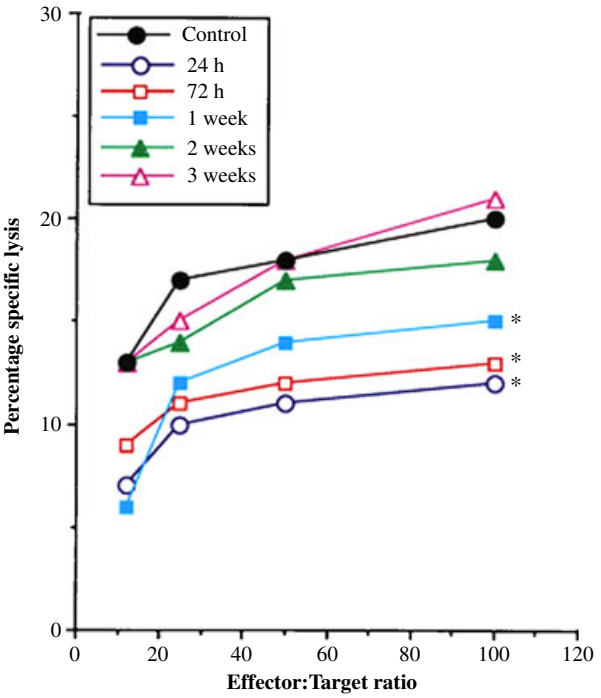

Figure 1 *In vivo* time–response study of a single dose of mAb D9 on splenic natural cytotoxicity (NC) of BALB/*c* mice (n = 20) measured *in vitro*. Mice were injected (i.p.) 40 μg of mAb D9. At different times (24 h, 72 h, 1 week, 2 weeks and 3 weeks) after mAb injection, five mice of each group were killed and their spleens were individually tested in ^{51}Cr release assay against WEHI-164 targets. Results showed a significant reduction in splenic NC activity within 24 h and persisted for up to 1 week (*$P < 0.05$, student's t test).

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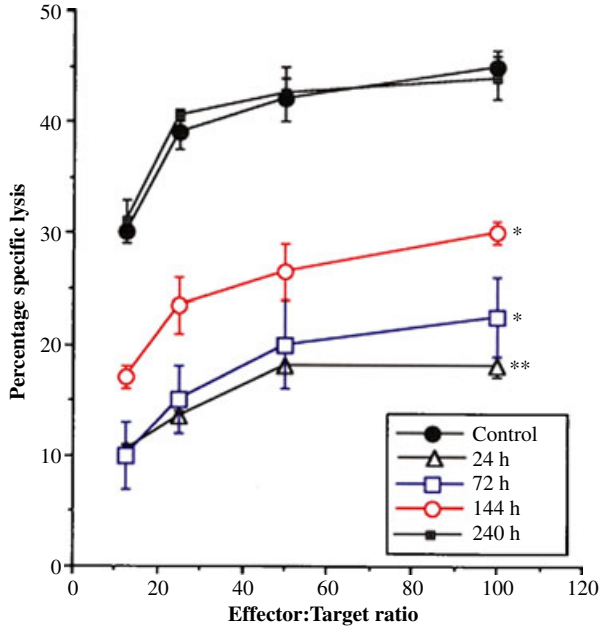

Figure 2 *In vivo* time–response study of a single dose of mAb D9 on splenic natural cytotoxicity (NC) of (CBA × C57BL/6) F1 mice (n = 20) measured *in vitro*. Mice were injected (i.p.) 40 μg of mAb D9. At different times (24 h, 72 h, 1 week, 2 weeks and 3 weeks) after mAb injection, five mice of each group were killed and their spleens were individually tested in ^{51}Cr release assay against WEHI-164 targets. Results showed a significant reduction in splenic NC activity at 24 h, 72 h and 1 week (**P < 0.001, *P < 0.05, students' t test).

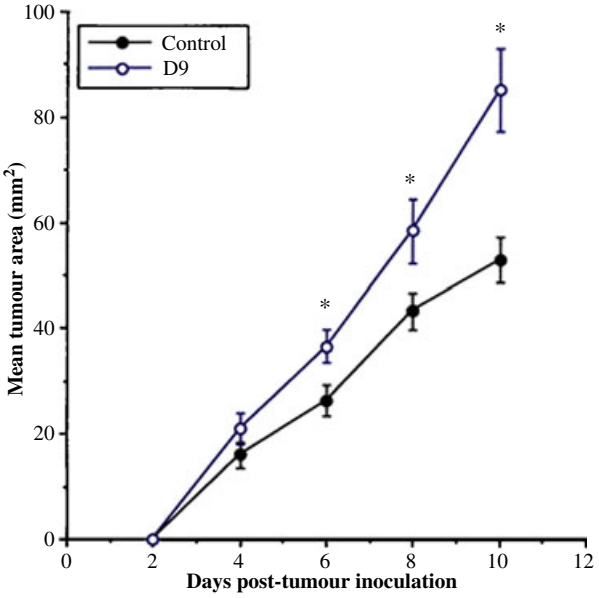

Figure 3 Effect of mAb D9 treatment on the growth of WEHI-164 fibrosarcoma in BALB/*c* mice. Between days 6 and 10 when the tumour area was <85 mm^2, mAb D9-treated mice consistently showed increased tumour growth compared to control mice treated with the isotype matched antibody. Results are presented as mean tumour area (mm^2) ±SE for groups of 25 mice (*P < 0.05, Mann–Whitney U test). The figure is the representative of two repeated experiments.

The difference in tumour growth was evident from as early as day 4 although the results did not reach statistical significance.

Role of NC-2+ cells on lung metastasis of WEHI-164 fibrosarcoma in BALB/c mice

Results of the experiment that examined the effects of mAb D9 on WEHI-164 metastasis showed significantly increased lung weights in the anti–NC-2 mAb-treated group when compared to control mice (the mice treated with tumour cells and PBS alone). Two weeks after WEHI-164 cell inoculation, the lungs of the mAb D9-treated group weighed between 0.243 and 0.940 g (mean = 0.543 ± 0.055) compared to the lung weights of the control group, which were between 0.175 and 0.385 g (mean = 0.268 ± 0.023) (P < 0.002). Number of tumour nodules in the lungs of the mAb D9-treated group was more than 100 and could not be counted. In contrast, the lungs of the control mice showed between 10 and 60 nodules (Fig. 4).

Discussion

Injection of a single dose of mAb D9 into mice depleted splenic NC activity within 24 h and the effect persisted for up to 1 week in a dose-dependent manner. *In vivo* tumour studies were undertaken to investigate the role of NC-2+ cells in surveillance against tumour growth and metastasis of the WEHI-164 fibrosarcoma. When syngeneic BALB/*c* mice were injected with mAb D9 and then challenged subcutaneously with WEHI-164, results showed significantly increased growth rate of the transplanted WEHI-164 fibrosarcoma when compared to control mice with normal NC activity.

As reported previously [21], incubation of spleen cells from a number of inbred mouse strains, BALB/*c*, C57BL/6, CBA and (CBA × C57BL/6) F1, with mAb D9 (anti-NC-2) reduced splenic NC activity against the NC-sensitive WEHI-164 target by approximately 50%, but NK activity against the NK-sensitive YAC-1 target *in vitro* was not affected [21]. BALB/*c* and (CBA × C57BL/6) F1 mice were selected according to our previous finding about the cytotoxicity of splenic leucocytes from different inbred mouse strains. The above-mentioned mouse strains had higher NC activity against WEHI-164 tumour cells. The cytotoxicity of (CBA × C57BL/6) F1 mice was more than other mouse strains, even more than BALB/*c* mice. This could explain why F1-mice Cr release assay results show a twice as efficient killing of target cells when compared to BALB/*c* mice.

The *in vitro* treatment also blocked effector spleen cell–WEHI-164 target cell conjugation by a similar amount. Further cellular, functional and biochemical data

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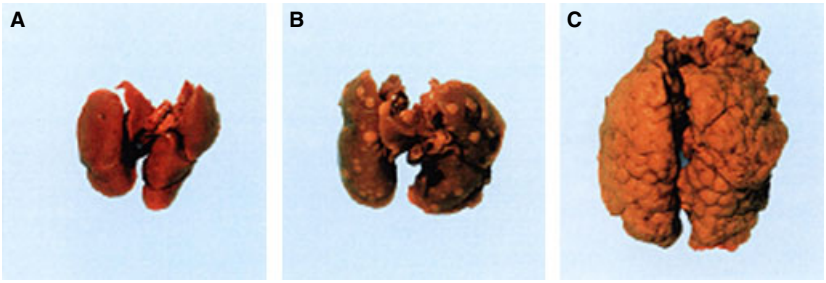

Figure 4 Photomicrograph of the lungs of BALB/*c* mice showing WEHI-164 tumour nodules. (A) No mAb treatment and no WEHI-164 inoculation; (B) PBS treatment and WEHI-164 inoculation; (C) mAb D9 treatment and WEHI-164 inoculation.

indicated that NC-2 is not the same receptor as the NC-1.1 receptor identified by moAb 1C4 on cells mediating NC.

Mice rendered NC deficient by injection of mAb 1C4 showed reduced tumour surveillance when compared to control mice [20, 22]. The *in vitro* data obtained for mAb D9 suggested that its injection into mice *in vivo* would also deplete splenic NC.

When WEHI-164 was intravenously transferred into BALB/*c* mice, a profound increase in metastatic lung nodules was observed in mice injected with mAb D9. The difference in lung weight was up to seven times greater in the mAb D9-treated mice than in the control mice, indicating a significant increase in metastasis. This is the first direct report that NC has a role in surveillance against metastasis and that this activity is mediated by both NC-2$^+$ cells.

The ability of NK cells in immune surveillance against cancer has extensively been reviewed by Waldhauer and Steinle [25]. NK cells exert their effector functions via exocytosis of perforin-containing cytolytic granules to directly kill target cells and the release of proinflammatory cytokines. The most important cytokines are interferon gamma (IFN-γ) and tumour necrosis factor. The activity of NK cells is mediated by a complex interaction between various inhibitory and activating receptors [26–29] signalling pathways initiated after cross-linking of the activating receptors resulting in the distinct effector functions are just beginning to be cleared.

A recent investigation showed that malignant cells express a set of ligands that mediate NK-cells recognition. These ligands are shared amongst murine cell lines, murine primary melanomas and human metastasis. These ligands recognition by NK-cell-activating receptors (NCRs and DNAM-1) mediates melanoma cell killing [30].

Experimental evidence was derived from a number of studies which showed that suppression of NK in mice resulted in an increased incidence of lung metastatic nodules [31]. It has been reported that allogeneic blood transfusion that reduced pulmonary NK also increased lung metastasis of an UV-2237 fibrosarcoma in syngenic mice, and this immunological surveillance was not because of T cells [32].

In conclusion, our data supports an innate surveillance in metastasis, at least of a chemically induced fibrosarcoma. It is possible that enhancement of NC by immunomodulatory drugs such as levamisole [33] could control the haematogenous spread of certain metastatic tumours.

It is not known whether mAb D9 blocks the NC-2 receptor after *in vivo* treatment or depletes a cell population that is mediating NC. This should be performed by immunophenotyping of spleen cells from D9-treated mice. Further study of the NC-2 receptor is also needed to characterize better the specificity of D9 antibody including information on different cell populations, the consequences of D9-injection on numbers of cytotoxic cell-population and cellular distribution in the spleens of mice. Multicolour flow cytometry to define the cell population that expresses the NC-2 receptor and co-staining with different markers of NK cells and other leucocyte/lymphocyte markers are also suggest.

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