Mouse embryos lacking RXRα are resistant to retinoic-acid-induced limb defects

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SUMMARY

Embryonic exposure to the vitamin A metabolite retinoic acid (RA) causes malformations in numerous developing tissues, including the limbs, which serves as a model system of retinoic acid action. RA treatment of wild-type mouse embryos results in digit truncations and long bone reductions. These effects are mediated by products of the retinoic acid and retinoid X receptor genes (RARs and RXRs), members of the nuclear receptor family of ligand-dependent transcription factors. Mouse embryos homozygous for a mutation in the RXRα gene appear normal in limb development, although such embryos are phenotypically affected in other tissues. We now describe resistance to limb malformations normally induced by teratogenic RA exposure in the RXRα−/− background. RA treatments that cause limb defects in 100% of wild-type embryos fail to elicit malformations in RXRα homozygotes, implicating RXRα as a component in the teratogenic process in the limbs. Heterozygous embryos are intermediate in sensitivity to RA, suggesting the importance of RXRα gene dosage in limb teratogenesis. Expression of the RA-inducible gene RARβ2 was equivalent between wild-type and homozygous embryos after RA treatment. RA treatment also did not distinguish between wild-type and RXRα−/− embryos in the spatial expression of sonic hedgehog (Shh) and hoxd-12, two other genes implicated in limb development. However, the quantitative level of hoxd-12 expression was elevated in RXRα−/− embryos. These observations indicate that transcriptional processes which are inappropriately regulated in the mouse limb by exogenous RA require RXRα for their execution, and that specific teratogenic processes, as well as specific normal developmental processes under vitamin A control, occur through individual members of the RXR and RAR families.

Key words: retinoic acid, RXR, limb defects, teratogenesis, gene dosage, mouse

INTRODUCTION

The vitamin A metabolite, retinoic acid (RA) is a critical regulatory signal molecule in numerous developmental and physiological processes. Experimentally, the biological requirement for RA has been elucidated through the analysis of phenotypes that emerge in animals after either exposure to exogenous RA, which causes inappropriate signaling, or to dietary vitamin A deficiency, which results in a failure of RA signaling for lack of its biosynthetic precursor. Numerous developing tissues, including the limb, are sensitive to RA deficiency, excess, or both (Warkany and Nelson, 1940; Kochhar, 1973, 1985), and the limb represents one particularly accessible model system of retinoic acid action. Each tissue has a unique temporal pattern of sensitivity to RA excess or deficiency, coincident with the time of morphogenesis. In all cases, the molecular mechanisms which result in these defects, and through which RA regulates these complex processes in normal development, have yet to be elucidated.

In the developing chick limb, a bead soaked in a moderate concentration of RA and placed in the anterior region of the early limb bud causes dramatic mirror-image duplications of the normal digit pattern (Tickle et al., 1982). This effect likely occurs through induction of an activity which is endogenously present in the posterior region of the limb, a domain termed the zone of polarizing activity, or ZPA. The response to RA is dose-dependent, and at high doses, where there is a uniform excess distribution of RA throughout the limb bud, truncations and deletions rather than duplications are noted (Tickle et al., 1985). In rodent studies, exogenous RA is delivered to embryos through maternal treatment and reaches uniform rather than localized levels through the limb. Truncations and deletions of the long bones (the humerus, radius and ulna in the forelimb), and digit deletions and fusions, occur after RA treatment of mammalian embryos (Kochhar, 1973, 1985). Lower doses result in a lower frequency and severity of limb defects, but duplications are only rarely seen in mammalian studies. Similar truncations are obtained in the chick by injection of RA into the amniotic cavity (Larsen and Janners, 1987), and in developing (but not regenerating) axolotl and Xenopus limbs by exposure to retinoic acid in the aquarium water (Scadding and Maden, 1986a,b), all of which result in
uniform exposures. The period of sensitivity to retinoic acid-induced malformation is at the limb bud stage, which in the mouse is between embryonic days 10.5 and 11.5 (Kochhar, 1973, 1985).

Embryonic vitamin A deficiency in mammals (Warkany and Nelson, 1940) results in a surprisingly similar spectrum of limb defects (reduction or deletion of long bones, and fused digits) as seen after exogenous RA treatment (Kochhar, 1973, 1985). The recovery of limb phenotypes following vitamin A deficiency demonstrates that endogenous processes utilize RA in the course of normal limb morphogenesis. Furthermore, the similarity in resultant phenotypes between RA deficiency and excess suggests that the consequences of each treatment might be attributable to a perturbation of the same signaling processes.

The effects of retinoic acid are mediated through specific receptors which belong to the nuclear receptor family of ligand-dependent transcription factors (which also includes receptors for steroid and thyroid hormones; Evans, 1988). Retinoid receptors form two distinct subfamilies of the nuclear receptor family, the RARs and the RXRs, each having three members α, β, and γ (Leid et al., 1992; Mangelsdorf et al., 1993). Retinoic acid signaling is mediated through several pathways: RXR-RXR homodimers (Zhang et al., 1992), heterodimers of RXR and RAR (Yu et al., 1991), and heterodimers of RXR with the orphan nuclear receptors NGFI-B, NURR1 (Perlmann and Jansson, 1995; Forman et al., 1995), and LXR (Willy et al., 1995). Each of these receptor complexes has unique biochemical properties, such as RA isomer specificity and DNA response element recognition.

Germline mutations of RAR and RXR genes have recently been established by us and others to further understand the role of the RA receptor genes in development. Simultaneous mutation of the RARα and RARγ genes (Lohnes et al., 1994) results in long bone shortening and in a variety of digit defects. The similarity of these phenotypes to the results of vitamin A deficiency described above indicates that the normal involvement of RA in limb morphogenesis occurs through RARα and RARγ, and presumably through a heterodimeric complex of these RARs with RXRs. Mutation of the RARα gene does not affect limb development, but does result in embryonic lethality around embryonic day 15.5 due to cardiomyocyte hypoplasia (Sucov et al., 1994; Kastner et al., 1994) and resultant decreased cardiac performance (Dyson et al., 1995). In this study, we have treated RXRα mutant litters with teratogenic doses of RA at or around embryonic day (E) 11.5. The timing of this treatment was such that limb defects were induced in wild-type embryos. Surprisingly, in RXRα homozygous embryos, no limb defects were apparent. These results indicate that RXRα is required for the induction of malformations induced by teratogenic doses of RA, but is not required for normal limb development, and illustrate a divergence in genetic requirement between normal and teratogenic limb development.

MATERIALS AND METHODS

Animals

The RXRα mutation used in this study has been described by Sucov et al. (1994). The transgenic RARβ2-β-galactosidase line was obtained with the construct RAR-PL-βGAL (Sucov et al., 1990), from which plasmid sequences were removed. All mice used in teratogenic and expression studies were inbred on the C57Bl/6 background (at least F2), with the exception of transgenic mice which were randomly outbred. RXRα heterozygous females were mated to heterozygous males (noon of the day of a vaginal plug defined as E0.5) and treated orally with 100 mg/kg all-trans retinoic acid (Sigma) suspended in sesame oil. Treatment for morphological studies was at E11.25-11.5, and for gene expression studies at E10.75-11.25. Embryos were cesarean isolated in PBS at appropriate times; yolk sac tissue was taken for determination of genotypes.

Visualization of limb morphology

For visualization of external morphology, embryos were fixed in 10% formalin in phosphate-buffered saline. For visualization of E14.5 cartilage elements, embryos were eviscerated, fixed in 95% ethanol for >6 hours, then stained overnight in 0.05% alcian blue (Sigma) in 90% ethanol/10% glacial acetic acid at 37°C. For visualization of E18.5 skeletal elements, embryos were ethanol fixed for >24 hours, and stained overnight in 0.05% alcian blue/0.017% alizarin red S (Sigma) in 90% ethanol/10% glacial acetic acid at 37°C. Skeletal elements were then rinsed with water, equilibrated in 20% glycerol, cleared in 20% glycerol/1% KOH at room temperature, and then stored in 20% glycerol. Ossified humerus lengths were measured with a reticle scale in a dissecting microscope at constant magnification.

Expression of marker genes

For β-galactosidase staining, embryos were fixed in 2% formaldehyde/0.2% glutaraldehyde/0.02% deoxycholate/0.01% NP-40 in PBS for 10 minutes on ice, then washed in PBS and stained at 30°C in 1 mg/ml X-GAL in 5 mM K3 Fe(CN)6/5 mM K4 Fe(CN)6/2 mM MgCl2 for 10 minutes on ice, then washed in PBS and stored overnight in 70% ethanol, then hydrated in water and cleared and stored in 50% glycerol. For whole-mount in situ hybridization, embryos were isolated at E10.5 or E11.5 and fixed immediately in ice cold 4% paraformaldehyde overnight. The following day, embryos were sequentially washed in PBS/methanol and stored in 100% methanol until use. Hybridization with digoxigenin-labeled riboprobes was as described by Izpisúa-Belmonte et al. (1993), and was visualized with alkaline phosphatase-coupled antibody (Boehringer) and AP-substrate (Boehringer). The mouse Sonic hedgehog (Shh) probe was a 350 bp sequence corresponding to the third exon, cloned by PCR amplification. The hoxd-12 probe (Izpisúa-Belmonte et al., 1991b) represents the carboxy-terminal coding and the 3’ untranslated regions of the mouse gene.

RESULTS

RXRα-/- embryos are resistant to limb defects induced by RA

The critical period for inducing limb defects in mice is between E10.5 and E11.5 (Kochhar, 1973; Kochhar, 1985). Pregnant RXRα heterozygous females mated to heterozygous males were treated with all-trans RA (100 mg/kg) between E11.25 and E11.5, and embryos isolated at E14.5 before the onset of homoygote lethality. This treatment is a relatively high dose, and results in limb defects in 100% of wild-type embryos. However, none of the homozygous littermate embryos displayed any such abnormalities (Table 1). At E14.5, the types of limb defects seen most easily are digit defects, as this time is before the long bones have become ossified. The most common defect seen in affected embryos was loss of one digit (50% of affected limbs), either by outright elimination or by proximal fusion of two phalanges into a common
digit. Other defects included loss of two or more digits (25%), syndactyly (fused digits) where separation of two or more digits (usually 2-3 or 2-3-4) was retarded or incorrectly executed (in 20% of affected limbs), and a rudimentary thumb (5%) (Fig. 1). The defects observed in this study are all known consequences of RA exposure. Furthermore, the teratogenic effects seen in this study were stochastic – different defects were often seen on opposite limbs of a given animal, and mildly affected pups were frequently found with littermates of the same genotype that were more severely affected. Defects were seen in both forelimbs and hindlimbs of wild-type treated embryos without any apparent bias, and without bias in handedness, but were never observed in untreated embryos of any genotype. Artefactual explanations for RA resistance as a secondary manifestation of the RXRα mutant phenotype (i.e., general developmental arrest, or failure of the administered RA to reach the limb) were not borne out by observation or experimental detection (see below). The resistance of RXRα-/-embryos to RA-induced limb malformations therefore implicates RXRα as a required component of the teratogenic process in the limb.

### Resistance to teratogenesis is subject to RXRα gene dosage

In contrast to the absolute responses of wild-type and mutant embryos to RA treatment, heterozygous embryos displayed an intermediate sensitivity, with a significant number appearing normal (Table 1). Furthermore, a greater proportion of heterozygotes than wild type exhibited only the relatively more mild defects of syndactyly, rather than digit deletion (Table 1), and affected heterozygotes were more prone to unilateral rather than bilateral defects (data not shown). This suggests that RXRα gene dosage is a contributing factor in transducing the teratogenic effects of RA, and that the RXRα mutation has a heterozygous phenotype in this assay.

To further verify the gene dosage requirements of RXRα in this assay, wild-type females were mated to heterozygous males, treated with RA at E11.25-E11.5, and pups isolated at E14.5. Limb defects were analyzed by external appearance and/or by alcin blue staining. Normal indicates five well-formed and separated digits, mildly defective indicates the presence of five digits but with either a rudimentary thumb or a defect in the separation of at least two of the digits (syndactyly), severely defective indicates at least one digit missing. Forelimbs and hindlimbs were considered equivalently in this analysis; embryos were scored on the basis of the most severe defect on any of their limbs.

### Table 1. Digit defects in RA treated embryos at E14.5

<table>
<thead>
<tr>
<th>RXRα genotype</th>
<th>Normal</th>
<th>Mildly defective</th>
<th>Severely defective</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>0</td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>5</td>
<td>11</td>
<td>23</td>
</tr>
<tr>
<td>Homozygous</td>
<td>9</td>
<td>0</td>
<td>0</td>
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9 litters (64 pups) derived from RXRα heterozygous mating pairs were treated orally with 100 mg/kg all-trans RA at E11.25-11.5, and pups isolated at E14.5. Limb defects were analyzed by external appearance and/or by alcin blue staining. Normal indicates five well-formed and separated digits, mildly defective indicates the presence of five digits but with either a rudimentary thumb or a defect in the separation of at least two of the digits (syndactyly), severely defective indicates at least one digit missing. Forelimbs and hindlimbs were considered equivalently in this analysis; embryos were scored on the basis of the most severe defect on any of their limbs.

In an effort to dissect the molecular pathways which might account for the teratogenic limb phenotype, and for resistance to malformation in the RXRα-/- background, we have looked at the expression patterns of three genes which have been implicated in normal and RA-exposed limb development.

Growth and differentiation of the mesenchyme in the developing limb bud occurs under the control of the overlying apical ectodermal ridge (AER). We (Sucov and Evans, unpublished data) and others (Mendelsohn et al., 1991; Reynolds et al., 1991) have previously established transgenic lines of mice expressing β-galactosidase under the control of the RARβ promoter, and observed RA-dependent expression of β-galactosidase activity in the AER of wild-type embryos. The RARβ promoter contains a RA response element in its promoter and is a primary target gene of RA action in an autoregulatory manner (deThe et al., 1990; Sucov et al., 1990). We have bred this transgene into the RXRα background and determined the resultant expression pattern. No β-galactosidase staining in the limbs is seen in untreated transgenic embryos of any genotype, verifying that expression is dependent upon induction by RA (Fig. 3A,B). In both wild-type and mutant transgenic embryos, treated with RA 6-8 hours earlier, equivalent staining is induced in the AER (Fig. 3C,D), in both forelimbs and hindlimbs. Thus, this marker of altered gene expression initiated by RA treatment remains properly regulated in the absence of RXRα. Furthermore, this result indicates that RA treatment is able to infiltrate into the developing limb and induce a transcriptional response in RXRα-/-embryos.

In the developing chick limb, a bead soaked in moderate levels of RA induces digit duplications when implanted into the anterior region (Tickle et al., 1982). This reorganization is preceded by the induction of genes expressed in the limb mesenchyme, including the HoxD gene complex (Izpisúa-Belmonte et al., 1991a) and the gene Sonic hedgehog (Riddle et al., 1993). A similar analysis of expression patterns of these genes in the mouse embryo exposed systemically to RA
expression pattern of hoxd-12 and Shh in wild-type and RXRα−/− embryos by whole-mount in situ hybridization. hoxd-12 and other members of the HoxD family are expressed in a broad overlapping region of the posterior dorsal limb bud, evolving from a straight to a curved pattern with advancing development of the limb. The spatial expression pattern of hoxd-12 was identical in wild-type and RXRα−/− littermate embryos, both without (Fig. 3E,F) and after RA treatment 23 hours earlier (Fig. 3G,H). (The differences seen between untreated and treated embryos shown in Fig. 3E-H reflect the slightly different developmental stages at which these embryos were isolated.) There was an increase in hoxd-12 staining intensity in mutant relative to wild-type embryos, but this was observed in both untreated and treated embryos. Shh is normally expressed in the posterior-distal region of the extending limb bud. As shown in Fig. 3I-M, the identical pattern of Shh expression was seen in wild-type and RXRα−/− embryos, both without and following RA treatment. For both hoxd-12 and Shh, and in both genotypes, expression is reduced from E12 onward and is ultimately extinguished, regardless of RA treatment (data not shown). Thus, in the mouse embryo, during the window of sensitivity to RA-induced malformations, the patterns of expression of these genes do not appear to change after RA treatment, irrespective of RXRα genetic background.

**DISCUSSION**

The observations reported in this paper indicate that limb malformations induced by teratogenic doses of RA are mediated through a pathway that requires RXRα. This observation is reminiscent of a RA-induced truncation of the spinal cord which requires the presence of RARγ (Lohnes et al., 1993), and supports the contention that specific teratogenic processes are mediated by specific retinoid receptors, as are specific normal processes.

**Are normal and teratogenic limb development genetically separable?**

It is clear that retinoic acid is an important agent in normal limb development. Vitamin A deficiency (Warkany and Nelson, 1940) and mutation of RAR genes (Lohnes et al., 1994) both cause a failure of normal RA signaling, and both result in similar limb deletions and truncations. Because the RARs function as heterodimers with RXRs, the active complex which mediates normal endogenous retinoic acid signaling is presumably a RXR-RAR heterodimer. If so, loss of RXRα is not sufficient to inhibit normal morphogenesis of the limbs, at least up to the time of embryonic lethality around E15.5. If RXRα is involved in normal RA signaling, our results would suggest that its function could be subverted by the remaining levels of RXRβ and/or RXRγ. We note in analogy that RARα and RARγ are both involved in limb development (Lohnes et al., 1994) with mutation of either alone not leading to a limb phenotype.

It might be imagined that RA signaling in normal and teratogenic limb development are separable molecular phenomena, in that RXRα is involved only in teratogenesis. Indeed, in addition to the RXR-RAR heterodimers which are involved in normal limb development, the RXRs are active in several additional pathways of RA responsiveness (as homodimers and as heterodimers with NGFI-B and LXR), any of which could be responsible for mediating teratogenic development. This might suggest that RA treatment induces a latent transcriptional process through RXRα which is qualitatively different from that induced by endogenous RA signaling and which is antagonistic to normal limb development. However, synthetic retinoids which are RXR selective are not teratogenic in the limbs and elsewhere (Jiang et al., 1995), whereas RAR agonists are very effective teratogens. Of the known pathways of RA responsiveness, only the RXR-RAR heterodimer is activated by RAR ligand occupancy. Teratogenesis therefore is most likely initiated by inappropriate activation of the RXR-RAR heterodimer, rather than through an alternative pathway.

At least two potential mechanisms might explain the obligatory involvement of RXRα in teratogenic limb morphogenesis, and its dispensability in normal development, even

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**Fig. 1.** Limb phenotypes of RA-treated E14.5 embryos. (A-F) Alcian blue stained forelimbs of three littermate embryos. Genotypes of these embryos are: (A,D) RXRα−/−; (B,E) wild type; (C,F) wild type. (G-I) Dark-field view of left hindlimbs of RA treated non-littermate embryos. Genotypes are: (G) RXRα−/−; (H) heterozygous; (I) heterozygous. Note the presence of five normally formed digits in RXRα−/− embryos (A,D,G), and various digit defects in heterozygous and wild-type embryos: digit deletion (B,E,F), distal digit fusion (B,F), incomplete separation of digits (I), rudimentary thumb (C,H).
Fig. 2. Long bone defects in E18.5 embryos. (A) Distribution of ossified humerus lengths in untreated wild-type (open squares; n=14), untreated heterozygous (filled squares; n=20), RA-treated wild-type (open circles; n=20), and RA-treated heterozygous (filled circles; n=34) embryos. Humerus length is described in arbitrary units; the plot indicates, for each group, the percentage of humerus lengths which are shorter than the indicated size. (B–E) Alcian blue/alizarin red S stained left forelimbs. (B,C) Untreated; (D,E) RA-treated; (B,D) RXRα heterozygous; (C,E) wild type. The embryos in B and C are littermates, as are those in D and E. These examples were chosen for being of median humerus length for their respective group. Ossified bone is stained red. Abbreviation: h, humerus.

Fig. 3. Expression of marker genes in untreated and RA-treated limbs. Expression patterns of: (A–D) RARβ2-lacZ transgene; (E–H) hoxd-12; (I–M) Shh. A–B, E–F, and I–K are of untreated embryos; C–D, G–H, and L–M are of embryos treated 6 hours (C–D) or 23 hours (G–H, L–M) earlier with RA. Genotypes are: (A,C,E,G,I,K,L) wild type; (B,D,F,H,J,M) RXRα−/−. Embryos in A and B, C and D, E and F, G and H, I and J, and L and M are littermate pairs. All embryos were isolated at E11.5 except for those in IJ, which were isolated at E10.5. Note that for the embryos in I and J, expression of Shh is only seen in the forelimbs; expression is not seen in the hindlimb bud until several hours later (K).
though both processes appear to be mediated by RXR-RAR heterodimers. In one, RXRs may be expressed in the limb only in areas not normally exposed to RA. RXRs may therefore not even be involved in normal limb development, but becomes active only after exogenous RA treatment. The spatial expression of RXRα at E11.5, however, has not yet been demonstrated as the level of expression is below detection by in situ hybridization (Mangelsdorf et al., 1992; Dolle et al., 1994). As an alternative model, RXRα may be expressed in the same tissue which responds to endogenous RA. Accordingly, normal limb development would require that RA signaling does not exceed a threshold above which malformations would occur. In untreated RXRα−/− embryos, the residual level of RA signaling through the remaining receptors would be sufficient to maintain normal limb development. However, the decreased receptor level would preclude excessive teratogenic response. While both models are consistent with the available data, clearly a greater understanding of the downstream transcriptional processes which are induced by RA in normal and in pathological limb development will help distinguish between them.

**RA-induced genes in normal and abnormal limb development**

One of the best-known paradigms of vertebrate limb morphogenesis has been the developing chick limb, in which a source of RA placed into the anterior region of the limb bud causes a general respecification of pattern, resulting in digit duplications. In mouse embryos, truncations and deletions are seen following RA treatment, rather than duplications. It is most likely that the differences seen in these systems reflect the differences in experimental approach: in mouse embryos, exogenous RA is distributed uniformly after maternal administration, while in the chick studies, exogenous RA is released locally from an implanted bead. At high doses, the localized effect of the bead in the chick is overwhelmed, generating essentially a uniformly high level of RA distributed across the limb bud. This treatment results in the same deletions and truncations as seen in mouse embryos (Tickle et al., 1985). Uniform RA exposure achieved by injection of RA into the chick amniotic cavity also causes truncations (Larsen and Janniers, 1987). Duplications in the chick are only seen with localized moderate doses of RA, where populations of cells within the limb bud are exposed to different levels of RA. Reasonably, it is the interaction between these different populations which ultimately causes respecification of pattern. It is presumed that these interactions mimick those that occur in normal limb development.

The duplications seen in the chick with moderate localized doses of RA are presaged by alterations in normal gene expression patterns, including ectopic induction of *Shh* and HoxD genes. In mouse embryos, and regardless of RXRα genotype, expression of the *hoxd-12* and *Shh* genes are not obviously affected by systemic RA treatment. Most likely, the alteration in expression of *Shh* and HoxD genes in the chick limb bud is an underlying molecular manifestation of the alteration in patterning which moderate localized RA treatment causes. *Shh* and Hox genes, therefore, are markers not of RA exposure but of the respecification of patterning which results in duplications. In mouse embryos, where systemic RA treatment does not alter patterning, the expression of these genes remains unchanged.

While the spatial expression pattern of *hoxd-12* was identical between wild-type and RXRα−/− embryos, the quantitative level of expression was substantially higher in mutant embryos, regardless of RA treatment. Expression of the 5’ Hox genes is reported to be supressed by RA (Simeone et al., 1991), suggesting that RXRα may be involved in this process. However, no limb alterations result from elevated *hoxd-12* expression in RXRα−/− embryos. Consequently, the significance of the increased *hoxd-12* expression in mutant embryos remains unclear.

It remains to be determined which genes are altered in expression in the mouse limb, following RA exposure, as part of the pathway which results in malformation. There are numerous known genes in mouse and chick that are expressed in the limb bud which are interesting or potential candidates for mediating normal and teratogenic limb development (Maden, 1994; Tickle and Eichele, 1944). It should be possible to evaluate these candidate genes as important in mouse limb teratogenesis, both by alteration in expression pattern following RA treatment, and by resistance to alteration in the RXRα−/− background. The RXRα mutation should therefore be useful in defining the molecular mechanisms which underlie teratogenic as well as normal limb development.

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