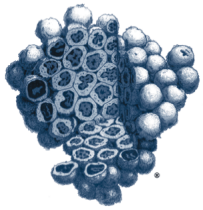

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On the Origin of Multiple Mutant Clones in Paroxysmal Nocturnal Hemoglobinuria

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Key words. Hemopoietic stem cells • Stem/progenitor cell • Stochastic model • Paroxysmal nocturnal hemoglobinuria

ABSTRACT

The pool of hematopoietic stem cells that actively contributes to hematopoiesis is small and the cells replicate slowly. Patients with paroxysmal nocturnal hemoglobinuria (PNH) invariably have a mutation in the *PIG-A* gene and many have more than 1 clone of *PIG-A* mutated cells. Typically there is a dominant clone and a smaller, second clone. By utilizing a combination of stochastic dynamics and models of hematopoiesis, we show that it is very unlikely that more than one *PIG-A* mutated clone arises at the level of the hematopoietic stem cells. More likely the smaller clone develops in the progenitor cell pool that would be expected to contribute to hematopoiesis for a shorter period of time. We provide estimates for the duration of these contributions and testable hypotheses that can shed important insights on this acquired hematopoietic stem cell disorder.

INTRODUCTION

Hematopoiesis is composed of a hierarchy of cell types that maintains the relative constancy of the number of circulating blood cells that are undergoing continuous turnover. The process is driven by the replication of hematopoietic stem cells (HSC) that have the dual capability of self renewal and differentiation into all types of blood cells [1, 2]. The size of the active HSC pool scales allometrically with adult mammalian mass and may be as low as ~400 cells in a healthy adult human [3]. The number of active stem cells is even smaller during human growth [4]. These cells replicate approximately 1/year [3, 5, 6] in an asymmetric fashion to renew themselves and provide cells to maintain downstream compartments throughout the hematopoietic cascade [7, 8].

Each time a HSC replicates, there is a small but finite risk of acquiring mutations in DNA. These mutations may lead to acquired genetic disorders such as hematopoietic tumors or paroxysmal nocturnal hemoglobinuria (PNH). The development of neoplasms has been described as the sequential acquisition of mutations by a cell that expands to form the malignant clone [9]. Given the increasing number of mutations that are observed in neoplasms, it has been argued that cancer is a disorder of genomic instability whereby cells have an intrinsically elevated mutation rate compared to normal cells [10, 11]. However, other models suggest that it is likely that the *initial* steps in the path to cancer are due to a normal mutation rate [12]. The normal

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mutation rate is 5×10^{-7}/replication [13] and the population of HSC is not only finite but small [3]. Therefore it is not surprising that stochastic effects play a determining role in the life history of a clone of cells [14]. The small size of the active HSC pool and the low mutation probability (in the absence of genomic instability) coupled with the rate of replication of these cells would suggest that acquiring more than 1 mutation in each cell is a highly unlikely event.

All patients with PNH have a mutation in the *PIG-A* gene that leads to a deficiency in the expression of GPI-anchored cell-surface proteins such as CD55 and CD59 [15]. Moreover, perhaps all healthy adults have a clone of variable size that has deficient expression of these GPI-anchored proteins [16]. Mutations in *PIG-A* occur at a normal somatic rate [17] suggesting that HSC in patients with this disorder do not have a mutator phenotype or genomic instability. The majority of *PIG-A* mutations correspond to small insertions or deletions that lead either to a shift in the open reading frame or to an early termination of the *PIG-A* product [18]. The result is the complete deficiency of GPI-anchored proteins (so called PNH type III cells). Less often, point mutations lead to an amino acid substitution within *PIG-A*. Such mutations can again result in complete loss of function and PNH type III cells or markedly reduce their activity with a significant reduction in expression of the GPI-anchored proteins on cells (PNH type II cells) [19]. Interestingly, many patients (if not all) with PNH, have more than 1 clone of mutated cells based on CD55 and CD59 expression by flow cytometry [20-22]. Moreover, recently two patients have been described with a defect on the short arm of chromosome 12 that may interfere with the function of the *HMGA2* gene [23] as well as an *independent* mutation in *PIG-A*. These investigators proposed that the mutation in HMGA2 is the mechanism behind clonal expansion of the PIG-A mutated cells. In view of the previous discussion, this should be a highly improbable event in such a small pool of HSC.

In order to understand these observations, we combined our work on the compartmental architecture of hematopoiesis [8] with stochastic dynamics [14]. The specific questions addressed in this work relate to: i) when can a second mutation in the same gene be neglected, ii) how does the structure of the hematopoietic system influence the appearance and relative contribution of mutants, and iii) what is the likelihood of a second independent mutation in a given cell within the hematopoietic cascade.

Mathematical Model and Results

A multi-compartmental model of hematopoiesis has been described in detail elsewhere [8]. Briefly, under steady state conditions, HSC in the active pool divide symmetrically with probability x and produce either two stem cells or two differentiated cells with equal probability. With probability 1-x, they divide asymmetrically giving rise to one cell for self-renewal and another cell that enters the pool of short-term repopulating cells. Subsequent rounds of division in downstream compartments k are coupled either with differentiation (and migration to compartment $k+1$) or self-renewal with both daughter cells staying within a given compartment k. Under steady state conditions, each compartment accommodates a number of cells, N_k. We used the principle of cell flux conservation to map out the size and rate of replication of cells in the various compartments of hematopoiesis. We obtain a progressive increase of compartment size and of the intrinsic replication rate of cells as they become more committed towards a specific cell lineage [8]. Within each compartment, cells divide at a rate r_k specific for each compartment. This rate increases as the cells become more differentiated. On the other hand, the mutation rate μ is the same across compartments. Since on average each compartment has a fixed number of cells, we shall model cell dynamics within each compartment in the form of a Moran process [24].

Two mutations within the stem cell pool

First we address the question whether two independent mutations in the same gene (e.g. *PIG-A*) can occur at the level of the stem cell pool before the disease is detected. The process is illustrated in Figure 1-A and B. In Figure 1-A a single HSC mutation (red cell) is responsible for 1 clone whereas in Figure 1-B independent mutations in different HSC (red and green cells) give rise to two clones. In the case of PNH, at least 20% of circulating neutrophils have to be deficient in GPI-anchored proteins for diagnosis [22]. We estimate the probability that a second mutation occurs in the stem cell pool while the first mutant clone increases to a size for which diagnosis is possible. Assuming Moran dynamics, the conditional average time until a mutant reaches the threshold M in a stem cell pool of size N can be calculated from the general equation for fixation times [25]. For *neutral* mutants, the average number of times each cell divides is given by

$$C = \frac{1}{x} \cdot \frac{N}{M} \sum_{i=1}^{M-1} \frac{M-i}{N-i}. \quad (1)$$

For $M = N$, this reduces to the fixation time of a neutral mutant in $N-1$ generations (one generation amounts to N Moran steps). In order to estimate how many mutations arise during these C cell divisions, we note that there are at most $N-1$ unaffected cells during the process. Hence, the maximum number of cell divisions occurring in this population cannot exceed $C \cdot N$. If the mutation rate per gene per cell division is u, then the upper limit for the expected number of new second mutants during the time until the first mutant reaches the threshold M is given by

$$F < \mu \cdot N \cdot C = u \cdot N \cdot \frac{1}{x} \cdot \frac{N}{M} \sum_{i=1}^{M-1} \frac{M-i}{N-i} \quad (2)$$

Assuming that each hematopoietic stem cell is equally represented in the circulation, with $N = 400$ [3], $M = 0.2 \cdot N = 80$, x=0.5 and $u = 5 \times 10^{-7}$ [13], then from equation (2), $F < 0.017$. Hence the probability that a second mutation in the same gene arises in another stem cell is below 2% suggesting that in PNH, one clone probably does *not* arise at the level of the stem cell (see discussion).

Simultaneous mutations in the stem cell and downstream compartments

In this case, we have to consider cell dynamics within and between compartments. Given the size of the hematopoietic organ and the turnover of cells [8], it is more likely that additional mutations occur in compartments downstream from the HSC compartment. This is illustrated in Figure 1-C where one clone (green cells) originates in a downstream compartment, while another clone (red cells) originates in the HSC compartment. Within any compartment (k) downstream of the stem cell pool ($k > 1$), in each time step, a cell is selected at random for reproduction. It can divide in two ways: (i) With probability ε, the selected cell gives rise to two differentiated cells that both move to the next compartment. This process decreases the size of the compartment by one ($N_k - 1$) and so the upstream compartment ($k-1$) replenishes the number of cells in compartment k to the constant value N_k that is characteristic for that compartment. (ii) The cell contributes to self renewal with probability $1-\varepsilon$ and in this case, a random cell from compartment k is chosen for differentiation and moves to the next compartment.

For simplicity, we consider the neutral case in which mutated cells replicate at the same rate as normal cells (normal fitness). We calculate the average time a mutation persists in a downstream compartment starting from i mutated cells. The probability that one of the i mutated cells is selected for reproduction out of a total number of N cells is i/N. The probability that this cell differentiates and leaves the compartment is $T^{-}(i) = \varepsilon \cdot i/N$. A new cell from the upstream compartment where no mutants are present replaces it. Thus, this step always reduces the number of mutants. On the other hand, the

number of mutated cells may increase due to self-renewal, with probability

$$T^+(i) = (1-\varepsilon)\cdot i/N \cdot (N-i)/N < (1-\varepsilon)\cdot i/N.$$

Self-renewal occurs with probability $1-\varepsilon$ and takes place in a mutated cell with a probability *i/N*. Moreover, in this case we have to choose one (normal) cell for differentiation in order to keep the compartment size constant. This occurs with probability $(N-i)/N$, as reflected by the last term. Interestingly, mutated cells are disadvantageous in this process, as

$$s(i) = \frac{T^+(i)}{T^-(i)} = \frac{1-\varepsilon}{\varepsilon}\frac{N-i}{N} < \frac{1-\varepsilon}{\varepsilon} < 1 \quad (3)$$

for $\varepsilon > 0.5$. Consequently, the fixation probability $\phi(j)$ for *j* mutants in a non-stem-cell compartment cannot exceed that associated with neutral mutants

$$\phi(j) < \frac{j}{N}. \quad (4)$$

For large populations, the probability that a single mutant reaches fixation $\phi(1)$, becomes very small. The average number of cell divisions in each cell until a single neutral mutation is lost again is formally given by

$$\tau = \frac{1}{1-\varepsilon}\left(\frac{\phi(1)}{1-\phi(1)}\right)\sum_{i=1}^{N-1}\sum_{m=1}^{i}\frac{1-\phi(m)}{m}\frac{N}{N-m}\prod_{l=m+1}^{N}\frac{1}{s(l)}$$
. (5)

For disadvantageous mutants, $\phi(j) < j/N$ (as in our case) and large *N*, we can determine an upper limit for this time, which is given by

$$\tau < \frac{1}{1-\varepsilon} Ln\left[\frac{\varepsilon}{2\varepsilon-1}\right]. \quad (6)$$

Here, $Ln[x]$ is the natural logarithm. A detailed mathematical description of these derivations will be reported elsewhere.

Mutations in the downstream compartment will ultimately die out due to the underlying structure of hematopoiesis, although the time until their extinction occurs can be long (see below). For normal hematopoiesis, we have estimated that $\varepsilon = 0.84$ [8]. Using this value in equation (6) we find that a mutant, on average, is only present for 1.32 generations within a given compartment. The replication rate in compartment k is $r_k = r_0 \cdot r^k$ [8], where r_0, the replication rate at the level of the stem cell, is once per year [3, 5] and $r = 1.27$ [8]. Thus, we can now calculate the average time a neutral mutant stays in each compartment. In the first non-stem cell compartment, this time is 380 days and decreases to 54 days in compartment 10 (Figure 2B). We must emphasize that these are averages and stochastically, a mutant cell that arises in one of these downstream compartments can persist for longer or shorter times. For larger compartments, however, the mutant population will quickly die out and the size of the clone will be small enough to be undetectable with current technologies.

Does the clone always arise from a single cell in an initial compartment?

Let us consider a case where we observe a circulating clone that comprises 1 % of all cells in the blood. Is it legitimate to conclude that such a clone is due to a single mutated cell present in a single compartment? To address this issue, we deduce the probability that a single neutral mutant reaches a certain threshold in a population. The probability that a second cell with the same mutation is produced within the same compartment is smaller than $1-\varepsilon$, which is 0.16 for the value of $\varepsilon = 0.84$ associated with normal hematopoiesis. If we assume the compartment has 10^3 cells, the probability that 10 (1%) cells of this type are produced in the compartment as a result of the original mutation is already smaller than 10^{-6}. Thus, we can conclude that usually, the mutant population does not reach a significant fraction of the compartment size. Notice that the size of compartment $k = 1$ is already of the order of 10^3 cells in normal hematopoiesis. Hence, circulating

mutant cells are truly clonal and maintained by a very small pool of cells in the bone marrow associated with the smaller compartments.

DISCUSSION

With the availability of sensitive diagnostic technologies such as flow cytometry and single cell polymerase chain reaction, circulating mutant populations are being found in many apparently healthy individuals [16, 26]. The hematopoietic system has a very high cell turnover [8] and so it is not surprising that even with a normal mutation rate almost every healthy individual can have mutated cells detectable in the circulation [16]. In PNH, perhaps the majority of patients have more than one distinct clone, although usually of different sizes [22]. In this work, we have attempted to understand the origin of the multiple clones that often occur in this disorder. We show that it is very unlikely for a patient to have two distinct mutations in *PIG-A* that arise in different hematopoietic stem cells. Our modeling suggests that the probability that this event happens is less than 1%. We point out that this is the probability for the case of a neutral mutant, the best case scenario since neutral mutants takes the longest time to reach a given threshold or ultimately fixation. If a mutation confers a fitness advantage, the time required for it to reach the diagnostic threshold will be shorter. This will reduce even further the probability that a second independent mutation in the same gene (e.g. *PIG-A*) will occur in another HSC. The facts behind this conclusion are the small size and slow rate of replication of cells in the active HSC pool [3] and the small (but known) mutation rate in the *PIG-A* gene [17]. Similar considerations suggest that it will be very unlikely that two mutations in separate genes (e.g. *PIG-A* and *HMGA2*) will occur in the same HSC. Therefore, we postulate that for the large majority of patients with PNH, mutations in *HMGA2* are not the reason for clonal expansion. Our results show that a second mutation in *PIG-A* is much more likely to occur in progenitor cells and this possibly explains the disparity in the size of the two clones. As illustrated in Fig. 1, this favors scenario 1-C as opposed to scenario 1-B. We expect that the clone arising within the HSC pool will be the larger one and it should persist for longer than the smaller clone which originates from cells within the progenitor pool (Figure 2). This prediction of the model is easily testable.

Our multi-compartment model of hematopoiesis suggests that multipotent progenitor cells (CFU-GEMM) are within compartments 5 to 8 [8] which would provide a total of ~150,000 cells, compatible with estimates reported elsewhere [27]. Most mutations in *PIG-A* observed in healthy adults are expected to occur in CFU-GEMM and more committed progenitors. Hence the small size and short survival of these clones in the circulation [16].

In summary, we have shown that multiple co-existent clones in the blood are unlikely to have their origin solely in the active HSC pool. The size of the clone and its survival in the circulation are related: on average, smaller clones will last for a shorter time, as they are associated with mutations in reasonably committed blood cell lineages.

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Figure 1. Potential evolutionary trajectories for multiple circulating clones. The first mutation occurs in the hematopoietic stem cell compartment (a) and the clone can expand on the way to reach the diagnostic threshold. The second independent clone can arise either due to a new mutation in another hematopoietic stem cell (b) or due to a mutation in a progenitor cell (c). Our analysis suggests that it is more likely that scenario (c) occurs rather than (b).

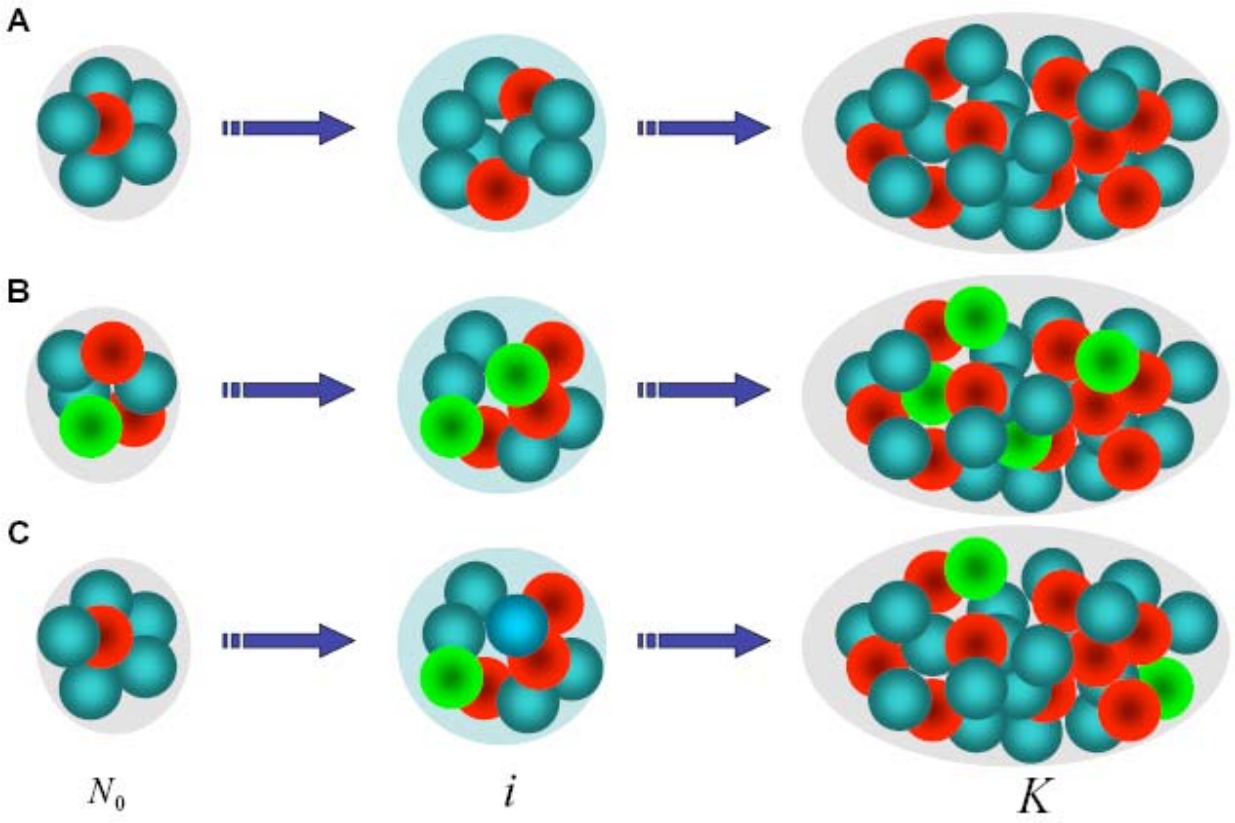
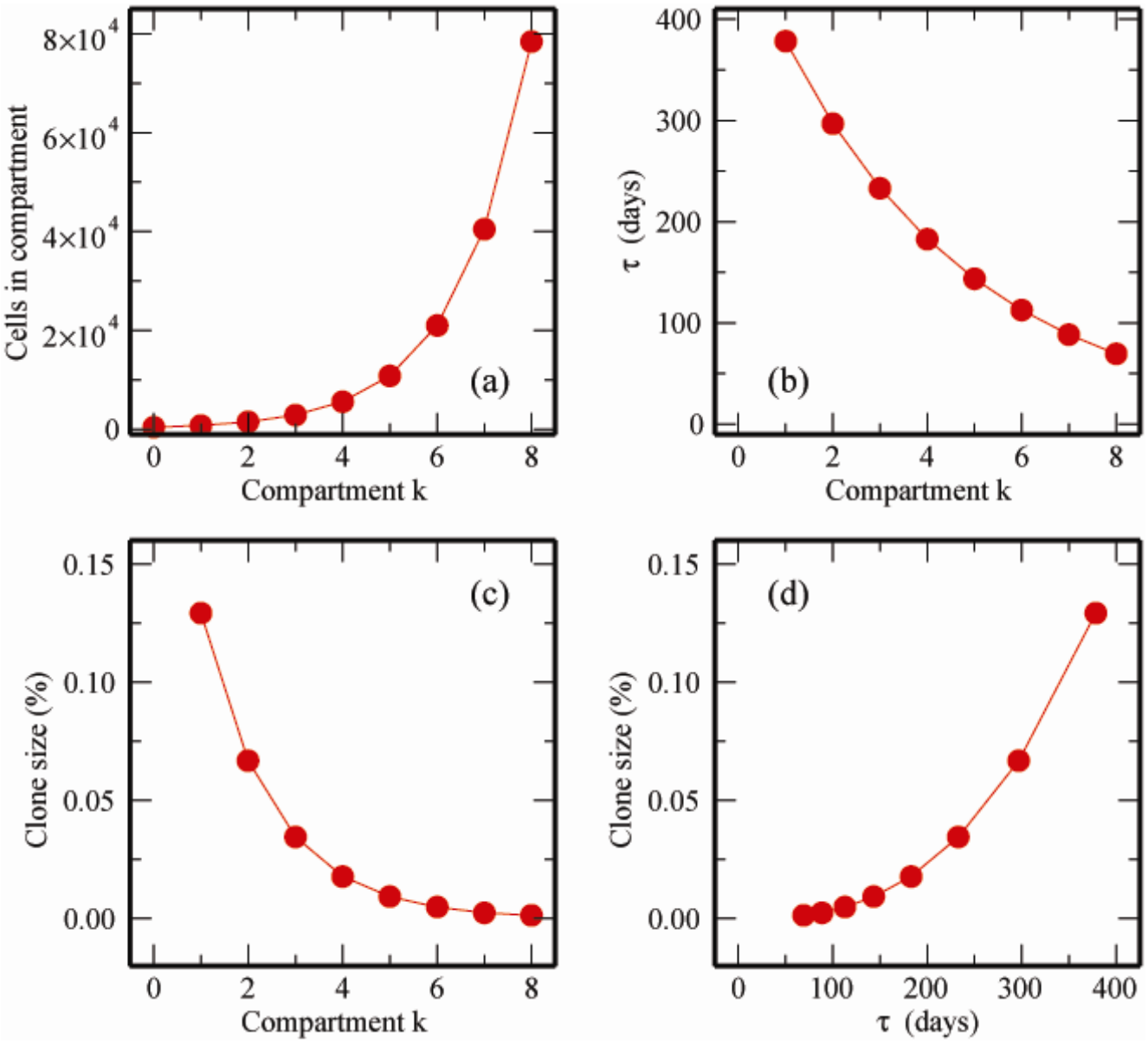


Figure 2. The compartment where the mutation appears, the size of the detectable clone and the duration of the clone in the circulation are intimately linked. As the compartment number *k* increases, (a) the size of the compartment increases exponentially; (b) the average time a mutant cell is present decreases and (c) the size of the circulating clone originating from that compartment decreases. Thus larger clones tend to persist for longer in the circulation (d).



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