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Divergent Molecular Pathways of Productive and Latent Infection with a Virulent Strain of Herpes Simplex Virus Type 1

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Mutants of herpes simplex virus (HSV) have been used to show that a variety of key genes associated with initiation of lytic infection or replication of viral DNA are not essential for establishment of latency. These observations are extended in the present study, in which a virulent strain of HSV type 1 that is not compromised in its ability to productively infect neurons under favorable conditions was used to demonstrate early divergence of molecular pathways leading to productive and latent infection. Our experimental strategy made unique use of the segmental innervation of the vertebrate trunk to study the spread of virus throughout the peripheral nervous system after inoculation of mouse flanks. Evidence of viral gene expression, including that of immediate-early genes, was transient, confined to ganglia directly innervating the inoculated skin (8th through 12th thoracic segments), and seen only at sites from which infectious virus could be recovered. In contrast, neurons containing latency-associated transcripts and reactivatable virus were more widely distributed (sixth thoracic through first lumbar segments), from which we conclude that replication-competent HSV type 1 can establish latency without initiating productive infection.

A characteristic of herpes simplex virus (HSV) is its ability to persist in the host in a nonreplicating (latent) state from which a productive infection may periodically reactivate. Despite the passage of two decades since Stevens and Cook (19) provided direct evidence that the viral genome persists in sensory ganglia, the molecular events that determine whether a neuron becomes productively or latently infected with HSV are poorly understood. A central issue is the nature of viral gene expression during establishment of latency.

Viruses that have been denied the possibility of initiating a lytic infection, for example, by deletion of an essential gene such as that encoding infected-cell polypeptide 4 (ICP4), retain the ability to persist in the host (12, 13), from which it has been concluded that the pathways of latent and productive infection are divergent from a very early stage. A corollary of this conclusion, taking into account the belief that replication of the HSV genome is dependent on several virally encoded proteins, is that viral DNA cannot be amplified during the establishment of latency. This corollary is not supported by indirect assessment of latent DNA copy number following infection with replication competent viruses. On the basis of the amount of DNA recovered from ganglia, it has been estimated that latently infected neurons harbor multiple (ca. 10 to 100) copies of the HSV genome (1, 4, 14). Consequently, deletion mutants may not accurately represent the behavior of replication-competent viruses, and the aim of this study was to determine whether a virulent strain of HSV type 1 (HSV-1) that is not compromised in its ability to enter the lytic pathway by a defect in its genome establishes a latent infection without accompanying viral gene expression.

Rather than manipulation of the virus, our strategy for restricting productive infection in neural tissue rested on careful selection of the experimental host. C57BL/10 mice were chosen because, like humans, they naturally limit the course of productive infection in spinal ganglia after inoculation of virus into the skin. By making novel use of the segmental cutaneous innervation of vertebrates, we precisely mapped the anatomical locations of viral gene expression and latent infection within the peripheral nervous system. In the acute phase of infection (during which latency is established), viral activity was assessed by quantitating infectious virus in homogenized tissue, immunohistochemical staining for viral antigens, and detection of viral mRNAs by in situ hybridization (ISH). At a later time point, established latency was detected by reactivation of virus in explant cultures and by the presence of latency-associated transcripts (LATs) in neuronal nuclei visualized by ISH (20).

Using this approach, we demonstrated the presence of latent infection in ganglia located at sites that show no evidence of viral gene expression during the establishment phase.

MATERIALS AND METHODS

Mice. Female C57BL/10 mice were obtained from the Specific Pathogen Free Facility, Animal Resource Centre, Perth, Western Australia, Australia. The animals were used at greater than 2 months of age.

Virus. Experiments were performed with a well characterized recent oral isolate of HSV type 1, strain SC16 (10). The pathogenicity of this virus has been carefully studied by various routes of inoculation with several mouse strains (9, 16). It is moderately neurovirulent after cutaneous inoculation and produces a transient phase of productive infection in spinal ganglia (16) followed by a stable latent infection that is indistinguishable at the molecular level from that seen in humans (4). The virus was grown and titrated in Vero cells and stored at −70°C until required.

Infection of mice. The model used in these experiments has been described previously (16, 18). Briefly, after depilation with Nair (Carter-Wallace, Frenchs Forest, New South Wales, Australia), a small patch of skin on the left flank defined by the spleen tip and corresponding to the 10th

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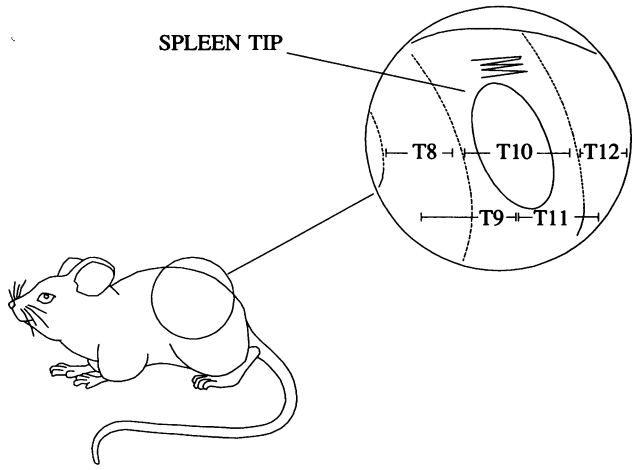

FIG. 1. Schematic diagram illustrating location of inoculation site. The enlarged area shows the area of scarification in relation to the spleen which is visible through depilated skin in the 10th thoracic dermatome (T10). Successive dermatomes overlap their neighbors by approximately 50%. Thus, T9 overlaps T8 rostrally and T10 caudally, and T9 and T11 meet around the middle of T10. Consequently, any scratch on the flank skin provides direct access to at least three sensory dorsal root ganglia.

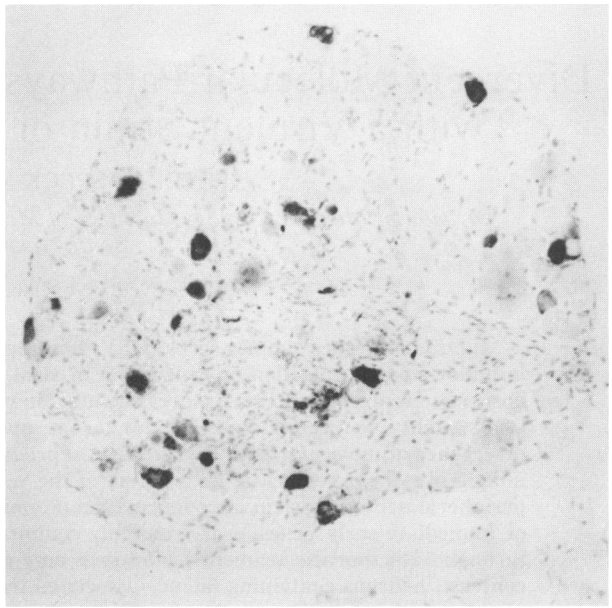

FIG. 2. Visualization of infected cells by immunohistochemical staining during acute ganglionic infection. HSV antigens (black areas) were almost exclusively confined to neurons, which were strikingly dispersed.

thoracic dermatome (T10; Fig. 1) was scarified with a 27-gauge needle by using a 10-μl drop of virus suspension containing 3×10^4 PFU of SC16.

Detection of infectious virus and latency. For quantitation of infectious virus, ganglia were homogenized in 1 ml of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered Dulbecco modified Eagle's medium (HDMEM), and 10-fold dilutions were tested by a standard plaque assay (11). To detect latency, ganglia were cultured in vitro at 37°C in 1 ml of HDMEM for 8 days, after which they were homogenized and tested for the presence of infectious virus.

Preparation of tissues for immunohistochemical analysis and ISH. Left sensory dorsal root ganglia (T6 to L1) were removed, fixed immediately in periodate-lysine-paraformaldehyde (8) for 60 min, and then transferred to 50% ethanol. Samples were paraffin embedded in pools of 30 to 40, representing either groups of mice killed at specific time points or individual vertebral levels. Five-micrometer-thick sections were collected onto glutaraldehyde-activated 3-aminopropyltriethoxysilane-coated slides.

Detection of viral antigens. The primary antibodies were (i) rabbit antiserum to HSV-infected cells (Dakopatts, Glostrup, Denmark), (ii) monospecific rabbit antiserum to ICP8 (a gift from Ken Powell, Wellcome Research Laboratories, Beckenham, United Kingdom), and (iii) monoclonal antibody (58-S; ATCC HB8183) to ICP4 (15). Binding of primary antisera was detected by using swine anti-rabbit or goat anti-mouse immunoglobulin as appropriate, followed by rabbit or mouse peroxidase anti-peroxidase conjugate, respectively (all from Dakopatts). All reactions were allowed to proceed for 30 min at 37°C with a 10-min wash in 50 mM Tris buffer (pH 7.4) between steps. Bound antibody was detected with 3,3'-diaminobenzidine (0.5 mg/ml, containing 0.1% H_2O_2), and sections were lightly counterstained with hematoxylin.

Detection of LATs and mRNA. Plasmid pBS-0, used to generate strand-specific riboprobes for detection of either LATs or ICP0 mRNA, consists of a 2,557-bp *Bam*HI-*Sal*I fragment of HSV-1 KOS cloned into Bluescribe M13$^-$ (Stratagene Cloning Systems, La Jolla, Calif.). Plasmid pBS-4, used to generate a strand-specific riboprobe for detection of ICP4 mRNA, consists of a 1,786-bp *Bam*HI-*Sal*I fragment from the ICP4 region of KOS, also cloned into Bluescribe M13$^-$. The plasmids were a gift from S. Efstathiou (Cambridge University, Cambridge, United Kingdom). The templates were linearized prior to transcription, and the probes were labelled to a specific activity of 3×10^8 dpm/μg with ^{125}I-CTP (NEN, Boston, Mass.).

The method used for ISH was a modification of that described by Gowans et al. (7). Tissue sections were hybridized overnight at 65°C with 1.6×10^2 pg of riboprobe. Unbound probe was removed by three sequential 2-h washes of increasing stringency up to T_m − 5°C. Bound probe was detected autoradiographically with LM-1 emulsion (Amersham, Little Chalfont, United Kingdom) by using a typical exposure time of 2 days. Sections were lightly counterstained with rapid hematoxylin.

RESULTS

Extent of acute and latent infection. The proportion of ganglionic cells expressing viral antigens was determined daily throughout the entire course of acute infection by immunohistochemical analysis of dorsal root ganglia (T6 to T13 pooled) removed from groups of 10 mice from the second to the seventh day after inoculation of virus into the skin. Ganglia were removed from an additional group of mice after 120 days and studied for the presence of LATs. Infection was largely confined to neurons (Fig. 2) and was detected first on day 4 (one neuron only), peaked sharply on day 5 (2.9% of neurons examined), and then cleared rapidly (Table 1). Antigen-positive neurons were notably clustered into a minority of the ganglia visualized in each section.

TABLE 1. Extent of productive and latent infection in C57BL/10 mice

Day after infection	No. of neurons[a]			No. of ganglia[a]		
	Examined	Antigen positive	LAT positive	Examined	Antigen positive	LAT positive
2	4,692	0 (<0.03)	NT[b]	34	0 (0)	NT
3	4,692	0 (<0.03)	NT	34	0 (0)	NT
4	5,934	1 (0.02)	NT	43	1 (2.3)	NT
5	9,936	285 (2.9)	NT	72	20 (28)	NT
6	6,348	72 (1.1)	NT	46	9 (20)	NT
7	3,174	4 (0.13)	NT	23	3 (13)	NT
120	17,940	0 (<0.006)	913 (5.1)	130	0 (0)	88 (65)

[a] Values in parentheses are percentages of total numbers examined.
[b] NT, not tested.

After 120 days, LATs (Fig. 3) were detected in 5.1% of neurons; the neurons containing LATs were scattered widely, such that the majority of ganglia examined (65%) showed evidence of latency.

The discrepancy between the proportion of latently infected ganglia and the proportion expressing viral antigens during the establishment phase suggests that the antigen-expressing cells are not essential precursors of the neurons that harbor long-term transcriptionally active viral genomes.

Anatomical distribution (T6 to L1) of viral gene expression during establishment phase. A more detailed analysis of viral activity at specific anatomical locations was undertaken. A further set of mice were infected within the region of the 10th thoracic dermatome, and 10 animals were killed at 24-h intervals between day 4 and day 7 in order to study ipsilateral dorsal root ganglia between T6 and L1 for evidence of viral activity (by immunohistochemical analysis for selected gene products or ISH for mRNAs). Fixed tissues were pooled with respect to vertebral level before being embedded in paraffin blocks. Forty mice were allowed to survive until day 45, at which time 30 were used to study the location of LATs and 10 were used for classical explant reactivation studies of individual ganglia. At the peak of infection (day 5), ganglia were removed from an additional three mice and tested for the presence of infectious virus, which was found only at the 9th, 10th, and 11th thoracic levels, peaking at T10 (Fig. 4a). Immunohistochemical analysis using a polyclonal antiserum directed against a wide variety of infected-cell polypeptides showed that the distribution of antigen-positive neurons was also restricted (Fig. 4b). Despite examination of multiple randomly selected sections (with typically 20 ganglia per section), we failed to detect any HSV antigen expression at T6, T7, or T12 to L1.

Specific evidence of expression of the immediate-early gene encoding ICP4 was sought because initiation of the HSV replicative cycle in cell culture is dependent on this gene. Both ICP4 and its mRNA were detected with apparently similar sensitivity by immunohistochemical analysis and ISH, respectively, at sites associated with productive infection, i.e., within the limits of T8 to T11 (Fig. 4c and d). mRNA of ICP0, which is partially complementary to the most abundant LATs, was detected with slightly greater frequency than ICP4 mRNA but in a similar pattern (Fig. 4e).

Replication of HSV DNA is thought to require the presence of the major DNA-binding protein ICP8, an early viral gene product (2). This protein could be readily detected immunohistochemically in the nuclei of infected neurons, but again its presence was restricted to ganglia between T8 and T11 (Fig. 4f).

We conclude that after inoculation of virus into the skin of C57BL/10 mice as described above, productive infection and detectable expression of ICP4, ICP0, and ICP8, and other immunogenic infected-cell polypeptides during acute infection (corresponding to the phase in which latency is established) are restricted to ganglia between T8 and T12.

Anatomical distribution (T6 to L1) of latency. Latent infection was widespread, such that in 30 mice killed for histological studies on day 45, neurons containing LATs were found at all sites examined (Fig. 4g). By using the classic approach of explant culture, virus could be reactivated from all levels tested (T6 to L1) (Fig. 4h); particular attention is drawn to T7 and T13, where a substantial proportion of ganglia became latently infected with strain SC16 despite the absence of detectable gene expression (including ICP4 and ICP0) and infectious virus at these levels during the establishment phase. We conclude that the molecular pathways of productive and latent infection with a virulent strain of HSV-1 can diverge from a very early stage.

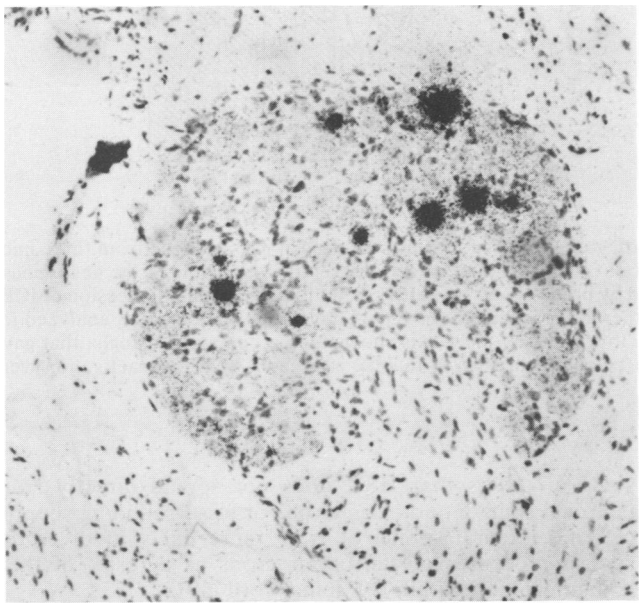

FIG. 3. Detection of LATs by ISH in a thoracic ganglion removed during latency. The abundance of LATs is illustrated by the dense clusters of grains typically found over neuronal nuclei after a 2-day photographic exposure (^{125}I-labelled riboprobe).

DISCUSSION

The studies reported here were made possible by the unique nature of the experimental model used. Segmental innervation of the vertebrate trunk, in the form of overlapping dermatomes (Fig. 1), provided an ideal system in which to monitor the spread of HSV from the skin to sensory ganglia that directly innervate the site of inoculation and from there to ganglia that innervate neighboring parts of the flank. We chose to infect mice in the 10th thoracic dermatome (T10), which is overlapped rostrally by T9 and caudally by T11 (5). It is also recognized that some neurons situated in the 8th and 12th thoracic ganglia might also send nerve fibers to the infected skin. Spread of virus from the skin directly to ganglia outside these limits is very unlikely.

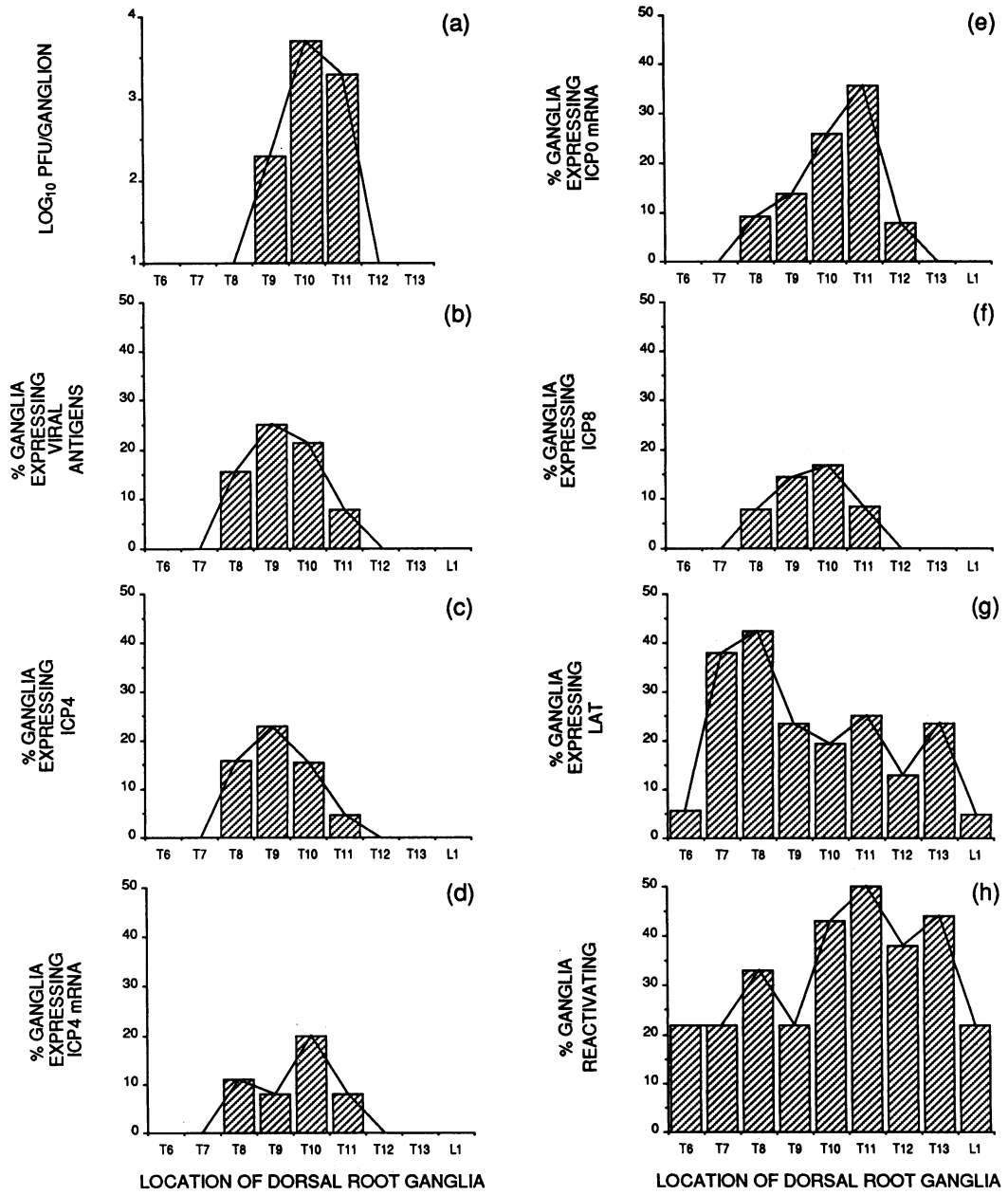

FIG. 4. Analysis of acute and latent infection in spinal ganglia (T6 through L1) after inoculation of virus at T10. Ganglia from three mice were tested for the presence of infectious virus at the peak of infection (a). Ganglia from 40 mice representing the whole of the acute phase of infection (days 4 to 7) were analyzed for expression of proteins detected by broad-spectrum anti-HSV antibodies (b) and expression of ICP4 (c), ICP4 mRNA (d), ICP0 mRNA (e), and ICP8 (f). Ganglia from a further 40 mice were removed 45 days after infection and analyzed for the presence of LATs by ISH (g) or reactivation by explant culture (h). In all graphs, the *y* axis shows the percentage of ganglia that gave a positive signal. Evidence of viral gene expression was confined to the region comprising T8 to T12, whereas LATs and reactivation were widespread.

During the acute phase of infection, the distribution of infectious virus and cells expressing viral genes correlated closely with the expected pattern of innervation of the inoculation site, confirming the power of HSV as a neurological tracer (21, 22). Within ganglia, infection was largely restricted to neurons, and indeed viral antigen was rarely detected in the capsular cells that surround each neuron. The widely scattered distribution of infected cells and the absence of syncytium formation in ganglia support the conclusions of others that HSV does not spread directly from neuronal somata to other neurons or to surrounding support cells (3). Neural spread of HSV more likely involves the central processes of sensory neurons as a result of their communication within the spinal cord (17).

Rapid, uncontrolled spread of HSV throughout the nervous system is a lethal complication of the model system described above when susceptible mouse strains (e.g., BALB/c) are used. The choice of C57BL/10 mice did not

entirely restrict the spread of the virus, but the outcome of infection in ganglia not directly innervating the inoculation site was establishment of latency, without apparent viral gene expression, rather than virus replication. The host factors responsible for restricting the replicative cycle are currently under investigation in our laboratory.

It is possible that, during the establishment of latency, viral proteins are produced in amounts not detected by either our immunohistochemical procedures or ISH or that an extremely short burst of gene expression was overlooked. However, because expression of ICP4 (an immediate-early gene), ICP8 (an early gene regarded as essential for viral DNA replication), and structural genes is represented in our analysis, we can conclude that if viral gene expression does occur, then from the earliest stages it is quantitatively and/or qualitatively different from that associated with productive infection. By demonstrating that a virulent strain of HSV can establish latency without detectably initiating the pathway associated with productive infection, our experiments confirm and extend observations made with viral mutants but leave unresolved the issue of how a strikingly large amount of viral DNA remains in latently infected tissue after resolution of a natural infection. We postulate that the bulk of this DNA is a residuum of aborted productive infection, perhaps unrelated to the transcriptionally active viral genomes in LAT-positive neurons. This hypothesis can be tested with the model system described in this article by determining the number of viral genomes present at each ganglionic level between T6 and L1 in latently infected animals.

Although generally within the same dermatome, the exact sites of primary and recrudescent herpes in humans are often different. For example, primary gingivostomatitis can recur periodically as cold sores (23), and herpetic whitlow in health care workers frequently recurs some distance from the point of initial infection (6). An extension of this phenomenon is perhaps seen in the model that we have developed, because latency was clearly detected in neurons that innervate regions of the flank far from the inoculation site. Establishment of latency by a virulent strain of virus at anatomically distinct sites uncomplicated by productive infection may be a useful system for studying the processes which determine the outcome of infection when a neuron is exposed to HSV.

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