Table 1. The case in which Human had been infected with rabies infection and symptomatic appearance.

	Place where Symptomatic sites were shown	Place of Infection site	Age (in years)
Overseas country			
July 2005	UK	India	Unknown
February 2005	Germany	India	Unknown
May 2004	Germany	India	51
June 2002	Taiwan	China	45
June 2001	UK	The Philippines	55
June 2000	Sweden	Thailand	19
Japan			
November 2006	Kyoto	The Philippines	69
November 2006	Yokohama	The Philippines	65
August 1970	Tokyo	Nepal	18

development of rabies [1, 3]. In 1885 in France, Louis Pasteur succeeded to save the life of a boy bitten by a rabid dog through sequential vaccination using attenuated rabies virus for the first time. Each year, provably 10 million people globally are vaccinated after exposure to rabies virus, e.g., by being bitten by rabies suspected animals [2].

Even in developed countries where canine rabies has been successfully controlled, cases of imported rabies are occasionally reported, in people shown symptoms of rabies after returning home who have been to rabies-prevalent areas and not been vaccinated after bitten by an rabies suspected animal [4]. However rabies is the infectious diseases of which outbreaks are becomes rare in the developed countries ex. such as Japan, UK, Germany, Taiwan, etc., people should still be properly informed about the risk and sufficient information of rabies in the endemic countries (**Table 1**).

Japan has been free of rabies for most of half century; the last case of indigenous human and animal rabies occurred in 1956 and 1957, respectively. And the last recorded case of imported rabies was in 1970 when a college student died in Tokyo after a trip to Nepal where he had been bitten on the right sural region by a stray dog. In November 2006, two cases of human rabies imported into Japan from the Philippines were identified. Two of late 60s were suspected of having rabies at the hospitals in Kyoto and Yokohama because of the presence of typical clinical symptoms and a history of bites by dogs in the Philipines. Saliva, cerebrospinal fluid, and skin biopsies (from the nape of the neck) were submitted for conventional ante-mortem diagnostic techniques.

In case rabies infection is suspected in humans, it is possible to prevent onset through an appropriate and rapid series of "Rabies PEP". The WHO recommends cleaning of the wound after the bite and post-exposure vaccination with successive injections (5 doses given on day 0, 3, 7, 14 and 30 and depending on the case, a 6^{th} dose on day 90). These injections are given during the long incubation period of rabies. However, unfortunately, three patients died of the imported rabies in Japan did not receive PEP after bitten by dogs in rabies endemic countries.

Because of the rarity of encountering rabies in Japan, Japanese travelers might be especially at risk and should be better informed [5]. For this reason, risk management of rabies is particularly important. And the experience of recent two imported rabies cases made clear that the risk of rabies in Japan is never zero. Anyone overseas bitten by animal suspected rabies should be treated PEP immediately and appropriately at the nearest medical facility. It is also important to consult with a doctor if bitten overseas and returning to Japan.

Governmental statistics in 2005 state that Japanese going abroad numbered 17,403,565 – an increase of 572,453 (3.4%) over the previous year. The number of people visiting Japan the same year was 7,450,103 – an increase of 693,273 (10.3%) compared to the previous year (the Ministry of Justice of Japan, white paper statistics[6]). Those traveling abroad are strongly advised to understand and know about rabies in countries they intend to visit. To familiarize themselves with species that transmit rabies, and to intentionally avoid contact with suspicious animals there are web sites such as the following: "Do you know zoonosis?"[7], "Association for the study of zoonosis"[8].

In general, in case of rabies, the virus excreted in the saliva penetrates the nerve tissues from the bite wound and the surface of the mucous membrane through the bite and it causes infection (**Fig. 2**). The Rabies moves slowly through the peripheral nervous system, which is thought to contribute to its long incubation period of anywhere from 1 to 3 months, to as long as 6 years. The rabies virus cannot be detected before its symptoms appear, but once they do, it may take less than one week from when symptoms appear to death, and the virus progresses quickly from the spinal cord to the brain and within the brain [1, 6].

When contracting rabies, the subject becomes extremely sensitive to external stimulation and shows a state of agitation. Animals will bite anything that moves in front of them (furious form). After onset, generalized paralysis occurs, and in the final stage, the subject or the

6. http://www.moj.go.jp/TOUKEI/index.html
7. http://www.forth.go.jp/mhlw/animal/
8. http://www.hdkkk.net/

Table 2. The death toll of Rabies and the estimated number of vaccinations after exposure (2004).

Country	Population (in millions)	Death	Postexposure vaccination
China	1,306	2,651	2,500,000
India	1,027	20,000	2,300,000
Indonesia	219	99	6,770
The Philippines	84	248	55,301
Sri Lanka	20	97	200,000
Thailand	63	19	351,535
Vietnam	82	81	615,000

Asian Rabies Expert Bureau (AREB) Meeting Report Vaccine 24: 3045-3049 (2006)

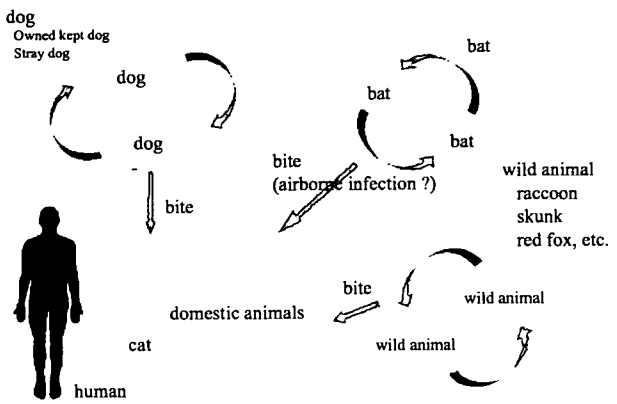

Fig. 2. Mode of rabies infection.

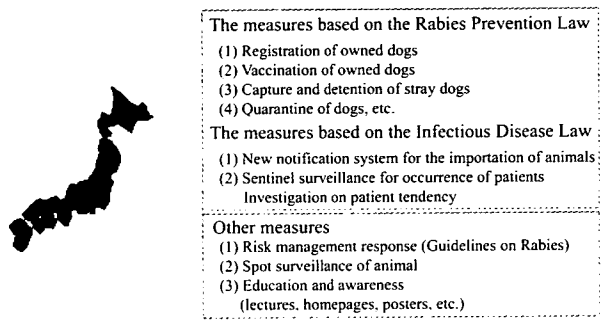

Fig. 3. Antirabies measures in Japan.

animals falls into coma and passes away. On the other hand, 15-20% of the animals display a constant state of paralysis (paralytic form). Hydrophobia is observed in humans. It is a condition in which the subject cannot drink water due to the pain caused by spasms of the pharynx, the larynx and the whole body when drinking [1, 2, 7].

According to a report by the WHO, over 90% of humans dying of rabies are found in Asia. Apart from that region, 500 to 5,000 people in Africa, 200-400 people in Latin America, 4-8 people in North America and 10-20 people in Europe are dying of rabies every year. The species of rabid animals differs with the countries and regions. The animals causing rabies outbreaks are mainly dogs (especially stray dogs) in Asian countries; dogs, jackals and mongooses in Africa; dogs, and bats (mainly vampire bats) in Latin America; raccoons, skunks, coyotes and bats in North America; and mainly foxes in Europe.

In Asia, it is vividly reflected in the following announcement issued by Chinese governmental health authorities: "The death toll from rabies far exceeds that of SARS or AIDS, making rabies the most fatal infectious disease in China." Rabies deaths in China increased dramatically from 1988, with 1,980 people dying of it in 2003 – an increase of 70% over 2002 –and over 2,600 dying of it in 2004 [8, 9]. In the Republic of Korea, canine rabies, once eliminated by 1984, broke out again in 1993 in areas bordering North Korea, and has since spread, claiming human victims in 1998 [5, 10]. **Table 2** lists shows the death tolls from rabies and numbers of people vaccinated after exposure in Asia.

In Japan, rabies has been culled out since 1957 thanks to the strong implementation of measures against rabies, such as vaccination of dogs, quarantine and control of wild dogs under the "Rabies Prevention Law" enacted in 1950 (**Fig. 3**). When the law was enacted, cases were reported in 54 humans, 867 dogs, and 29 cats. After the law mandated, during seven years, the endemic dog rabies was successfully eliminated in Japan [10–12]. Nonetheless dog rabies continues to prevail in Asian neighboring countries. Vaccination of dogs every year (April-June) constitutes one of the important measures for the prophylaxis of rabies [10].

Various infectious diseases may well penetrate into Japan in the coming years due to scenarios unthought-of so far or unpredictable routes of transmission. Over the last few years, the risk of imported infectious diseases has become a reality, as shown by the unexpected outbreaks of H5N1 bird flu and BSE in this country, the movements of SARS-infected travelers, the importation of prairie dogs suspected of being infected with Tularemia (a bacterial zoonosis caused by *Francisella tularensis*), etc. It is well-known as any route either unseen or unnoticed, becomes a hidden risk, which is why measures against outbreak are so important.

In a country where rabies is rare, such as Japan, the con-

(a)

☆ Proper understanding of rabies

☆ Provision of informations to traveler for the human who makes voyage to the rabies-risk regions

☆ Immediate vaccination after exposure due to bites, etc., in rabies-risk regions

(b)

☆ Decrease in import of infected animal quarantine, etc.

☆ Proper understanding of rabies and appropriate breeding

☆ Risk awareness of rabies import and outbreak

Fig. 4. (a) Rabies prevention in humans, (b) Rabies prevention in pets.

cern exists of whether an outbreak could be responded to effectively, swiftly, and appropriately [5, 10, 13]. Inappropriate or delayed response even to suspicious cases could lead to social anxiety. It is thus important to validate the existing measures, rabies prevention law, and implement effective measures with the monitoring. In this situation, it is desired to continue the research on potential rabies risk and undertake the appropriate measures against rabies [5, 10, 13]. Rabies prevention programs should be implemented that ensure public awareness and recognition of rabies and its potential effects, together with appropriate response (**Fig. 4**).

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Academic Societies & Scientific Organizations:

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- The Japanese Society for Bacteriology (JSB)
- The Japanese Society for Virology (JSV)

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特集　最近話題のウイルス

3. 狂犬病ウイルスの病原性に関する研究の進展

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狂犬病ウイルスは，人および動物に致死的な神経症状を主徴とする狂犬病を引き起こす．ワクチンによって効果的に予防できるにもかかわらず，本病の世界的な流行状況は好転していない．安価で安全な弱毒生ワクチンの開発ならびに治療法の確立が本病の制圧の鍵である．この目標を達成するためには，狂犬病ウイルスの病原性発現機序を解明することが重要となる．本稿では，狂犬病ウイルスの病原性に関する現在までの研究について紹介し，これをどのように狂犬病の制御に応用するのかについて考察する．

はじめに

狂犬病は，致死的な神経症状を特徴とするウイルス性人獣共通感染症である．治療法は未だに確立されておらず，発症した人および動物はほぼ100％死亡する．長く不定な潜伏期（6～150日，平均1ヶ月）が本病の特徴であり，極めて稀ながら6年の潜伏期の後に狂犬病を発症した患者の例も報告されている[29]．本病の病原体である狂犬病ウイルスは非常に広い宿主域を持ち，すべての哺乳類に感染すると考えられている．その中でも犬は，発展途上国における主要な病原巣となっている．一方，先進国ではキツネ，アライグマ，スカンク，コウモリなどの野生動物がウイルスの感染環の形成に重要な役割を果たしている．本病を発症した動物の多くは狂躁状態となり，他の動物個体に咬傷を加える．通常，発症動物の唾液には多量のウイルスが存在し，その咬傷口からウイルスが侵入することにより感染が成立する．様々な動物が人への媒介動物になりうるが，最も重要な動物は犬である．実際に，世界における人の症例の99％以上が犬の咬傷事故に起因すると言われている[39]．

我が国では，犬の予防接種，放浪犬の捕獲，動物の輸入検疫などの継続的な努力の結果，1957年における猫の1例を最後に狂犬病の撲滅に成功した．しかし，アジア諸国を含む世界中のほとんどの国においては，狂犬病は未だに大きな社会問題である．WHOの報告によると，発展途上国を中心に毎年55,000人以上が狂犬病によって死亡していると推定されている[38]．このような状況と近年の急速なグローバル化から，日本への本病の侵入もしくは輸入症例が発生する可能性が指摘されていた．2006年11月，不幸にもその懸念が現実となった．フィリピンから帰国した日本人が狂犬病を発症し，死亡するという事例が，2件立て続けに起こった[30, 42]．これらの事例は，1970年にネパールから帰国した旅行者が死亡した事例に続く，36年ぶりの狂犬病の輸入症例となった．このような輸入症例の発生は，狂犬病の撲滅に成功した日本であっても，本病の流行発生が身近な出来事であることを我々に強く印象づけた．これからは，「世界的な狂犬病の撲滅が，最も効果的な防疫手段である」という観点から，我が国の狂犬病に対する防疫を，さらに本病の対策に先進国が果たすべき国際貢献について考えて行く必要があるのではないだろうか．

狂犬病は，ワクチン接種により予防できる感染症である．また，咬傷によるウイルス暴露を受けた直後から集中的なワクチン接種（暴露後免疫）を行えば，ほとんどの場合，発症を予防できる．にもかかわらず，なぜ，世界中で狂犬病の流行に歯止めがかからないのだろうか？　その一因として，現行の不活化狂犬病ワクチンには多量の抗原が必要なため，ワクチンがコスト高となってしまうことが挙げられる．経済的理由により，このコスト高が本病の発生が集中している発展途上国でのワクチンの普及を困難なものにしている．一方，一般的に不活化ワクチンよりも安価な弱

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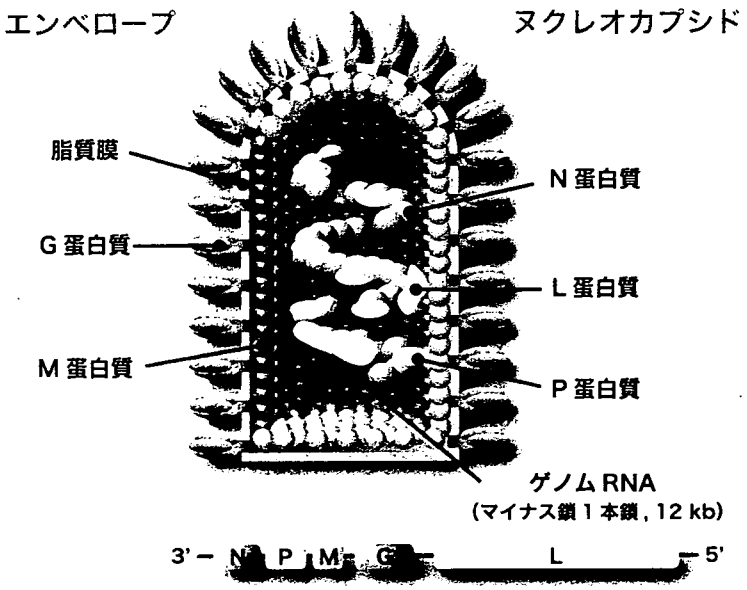

図1 狂犬病ウイルス粒子の構造

毒生ワクチンには安全面の懸念があるため，犬での使用は難しく，人への応用も不可能である．狂犬病を撲滅するためには，安全性の高い弱毒生ワクチンの開発が必要不可欠であると考えられる．

同時に，狂犬病に対する治療法が確立されていないことも本病の犠牲者が減少しない理由である．狂犬病ウイルス感染によって引き起こされる脳炎は，通常，顕著な神経細胞の変性・壊死を伴わないことが知られている．したがって，狂犬病発症時の激烈な神経症状は，脳の器質的変化によるものではなく，むしろ機能障害によるものと考えられている[14]．再生能の極めて低い中枢神経系が破壊されないという事実は，神経細胞の機能障害さえ除去できれば，狂犬病の治癒が可能であることを暗示している．しかし，ウイルスがどのように神経細胞機能を障害しているのか，ウイルスがどのように宿主の免疫を回避しているのか等，本病の治療法を確立する上で必要な狂犬病ウイルスの病原性発現機序に関する情報は未だに十分とは言えない．

狂犬病ウイルスの病原性発現機序を解明することは，安全，効果的かつ安価な弱毒生ワクチンの開発，ならびに狂犬病の治療法の確立の両面において極めて重要である．本稿では，これまでの狂犬病ウイルスの病原性に関する研究がどのように展開してきたかについて概説する．さらに，これまでに得られた研究成果をどのように狂犬病の制御に応用していくのか，その具体的な方向性についても考えてみたい．

狂犬病ウイルスの構造と分類

狂犬病ウイルスは，モノネガウイルス目ラブドウイルス科リッサウイルス属に分類される．ウイルス粒子は，幅60-110 nm，長さ130-250 nmの特徴的な弾丸状の形態をとり，その構造はエンベロープとヌクレオカプシドに区別される（**図1**）[40]．エンベロープは，宿主細胞由来の脂質膜とその表面にスパイク状に突出する糖(G)蛋白質ならびに膜の内側に局在するマトリックス(M)蛋白質によって構成される．G蛋白質は，受容体結合蛋白質であると同時に，pH依存的な膜融合活性を持ち，ウイルスの細胞内侵入時にエンベロープとエンドソームの脂質膜を融合させる．また，本ウイルスの感染防御に中心的な役割を担うウイルス中和抗体の誘導に関与することが知られている．M蛋白質は，子孫ウイルスの出芽および粒子形成に重要な役割を担っている．一方，ヌクレオカプシドは，ゲノムRNAとそれを包み込む核(N)蛋白質，リン酸(P)蛋白質およびL蛋白質から構成される．N蛋白質はヌクレオカプシドの形成に関与し，L蛋白質は，その共因子であるP蛋白質と共に，RNA依存性RNAポリメラーゼとしてウイルスRNAの転写・複製を担当する．ウイルスゲノムは，全長約12,000塩基，マイナス鎖1本鎖のRNAで，3'末端よりN，P，M，GおよびL遺伝子の順に上記の5種類の構造蛋白質をコードしている．

狂犬病ウイルスは，いわゆる野外流行ウイルスである街上毒と，ワクチン株や実験室株などの固定毒に大別される．固定毒はパスツールによって初めて確立され，街上毒をウサギやその他の動物の脳内に長期継代することにより作出される．最初の狂犬病ワクチンの開発の礎となった歴史的経緯を持ち，現在もワクチン製造や基礎的研究に広く用いられている．固定毒という名前は，街上毒の感染時に認め

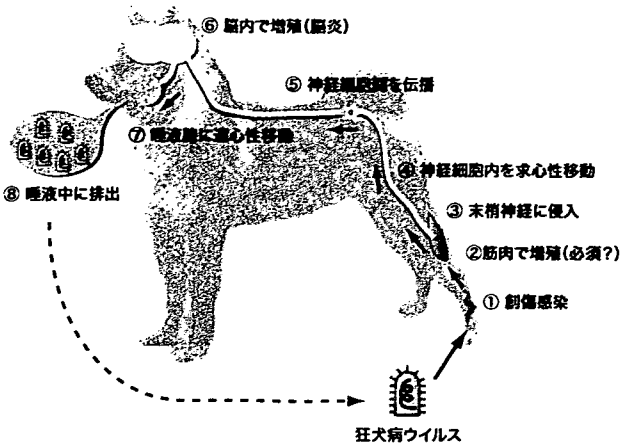

図2　狂犬病ウイルスの体内動態

られる不定な潜伏期が固定毒の感染では一定となることに由来している．また，街上毒に比べて著しく末梢感染性が減弱していることが固定毒の大きな特徴である．そのため，安全性の観点から街上毒はBSL3実験室で扱われるのに対し，固定毒はBSL2実験室での取り扱いが可能である．

末梢感染性を減弱した固定毒でも，成熟マウスへの脳内接種によって街上毒と同様に致死的神経症状を引き起こす性状を保持するウイルスが存在する．一方，このような固定毒を鶏胚や各種培養細胞で長期継代することにより，脳内接種によって致死的感染を起こさないウイルスも確立されている．本稿では，前者を強毒の固定毒，後者を弱毒の固定毒と定義する．

謎ばかりの狂犬病ウイルスの病原性

図2に狂犬病ウイルス（街上毒）の感染動物体内における動態を示した．まず，感染動物の唾液中に含まれるウイルスが創傷感染する．次に，創傷部位のウイルスは末梢神経に侵入し，神経線維を求心性に移動する．やがてウイルスは中枢神経系に到達し脳炎を誘起すると同時に，感染動物に神経症状を引き起こす．その後，ウイルスは神経線維を介して唾液腺を含む全身局所に遠心性に移動し，最終的に唾液中にウイルスが排出されるようになる．

上記の創傷感染の成立から脳炎の発症までのすべてのステップ（**図2**，①～⑥）が狂犬病ウイルスの病原性に密接に関わると考えられる．しかし，これらの機序は，ほとんど謎のままである．例えば，長く不定な潜伏期の間，ウイルスが感染動物の体内のどの部位にどのような構造をとって潜んでいるかは未だ不明のままである．また，この潜伏期の間，血中の狂犬病ウイルスに対する抗体はほとんど上昇しない．この間，ウイルスがどのように宿主の免疫を回避しているのかについても明らかになっていない．また，狂犬病ウイルスはニコチン性アセチルコリン受容体を介して筋細胞に感染することが知られているが[17]，筋細胞におけるウイルスの増殖が末梢感染の成立に必須か否かについても不明のままである．ウイルスの末梢感染経路として，主に運動神経が関与することが示唆されているが[15, 34]，感覚神経が関与する可能性も残されている．

狂犬病ウイルスの体内動態，特に末梢感染に関する機序が未だに解明されていない理由として，これまでの狂犬病ウイルスの研究が安全性の高い固定毒を利用して行われてきたことが挙げられる．大量の固定毒を末梢組織に接種（筋肉内投与，皮下投与など）すると，固定毒であっても末梢感染が成立することが知られている．この場合，接種後24時間には既に脳幹にウイルスRNAが検出されることから[26]，固定毒は末梢組織の非神経系細胞で増殖することなく直接末梢神経に侵入すると考えられている[14]．しかし，末梢感染性が減弱した固定毒による実験系が街上毒の末梢感染性を完全に再現しているとは考えがたい．

街上毒を一般的な実験動物に接種しても長く不定な潜伏期を再現することが難しいことも研究の障害となっている[4]．Baer と Cleary[3] は，ボブキャット由来の街上毒をマウスの後肢足蹠に接種し，17～120日の潜伏期を再現することに成功している．このモデル系により，長い潜伏期の間，ウイルスは神経細胞に侵入することなく，末梢の感染部位の近くに留まっていることが示唆されている．同様に，野生動物を用いた実験において，スカンク由来の街上毒をスカンクの筋肉内に接種することにより，潜伏期の間，ウイルスが筋細胞に感染していることを示唆する成績が報告されている[6]．このように，若干の知見はあるものの，街上毒の末梢感染機序の全容は未だに明らかにされていない．街上毒の特性である長く不定な潜伏期を再現できる実験動物モデルの確立が，機序解明の鍵となるであろう．

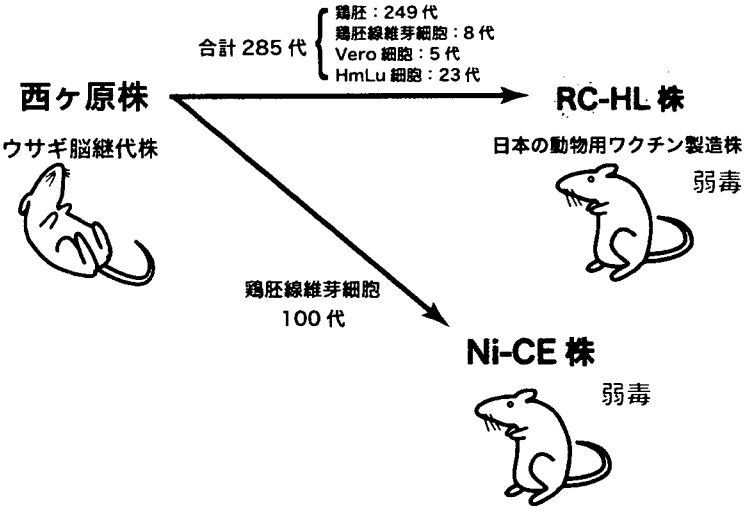

図3 西ヶ原株，RC-HL株およびNi-CE株の関係と病原性

狂犬病ウイルスの病原性決定因子

街上毒が引き起こす致死的な神経症状については，強毒の固定毒を成熟マウスに脳内接種することにより，再現することが可能である．この実験モデル系は，狂犬病ウイルスが脳内に到達した後に神経症状を引き起こすステップ（**図2**，⑥）の解明のために利用されてきた．これまでに，本モデル系を用いて，ウイルスの病原性決定因子の同定に関する研究が行われている．1983年，Dietzschold ら[8]は，抗G蛋白質モノクローナル抗体を用いて作出した強毒の固定毒株（CVS株およびERA株）の中和耐性変異株の中に，成熟マウスに致死的な感染を起こさない弱毒化したウイルスが含まれていることを見いだした．この弱毒変異株では，親株のG蛋白質333位のアルギニンがイソロイシンまたはグルタミンに変異していることが示された．他の研究者による追試もあり，G蛋白質の333位にアルギニンまたはリジンを持つウイルスは強毒に，それ以外のアミノ酸を持つウイルスは弱毒となることが明らかにされた[25,35]．ひとつのアミノ酸置換がウイルスの病原性を劇的に変化させる事実は，狂犬病ウイルス以外のウイルス研究分野にも大きな反響を与えた．その後の研究により，G蛋白質の333位に弱毒型のアミノ酸を持つ変異株は，神経系培養細胞への吸着および侵入，ならびに同細胞におけるCell-to-Cell感染の効率の点で強毒の親株よりも劣ることが示された[7]．また，感染マウス脳内における弱毒変異株の感染の広がりは，親株と比較して限局していることも明らかとなった．その後，狂犬病ウイルスの感染性cDNAが確立されると[23]，上記のような中和耐性変異株を用いなくても直接的に遺伝子を改変したウイルスの病原性解析が可能になった．感染性cDNAを用いた研究により，他のウイルス株でもG蛋白質333位のアミノ酸がウイルス病原性の決定に重要であることが確認され[19,31,41]，普遍的な病原性決定因子であることが示されている．

しかし，G蛋白質333位のアミノ酸が唯一の病原性決定因子でないことも予想されていた．日本の動物用狂犬病ワクチンの製造株であるRC-HL株は強毒の固定毒である西ヶ原株を親株とし，鶏胚および各種培養細胞で合計285代継代した後に確立された弱毒のウイルスである（**図3**）[10]．その弱毒性状にも関わらず，RC-HL株のG蛋白質333位のアミノ酸は強毒型のアルギニンであることが報告されていた[11,12]．各遺伝子の推定アミノ酸配列を西ヶ原およびRC-HL株間で比較すると，G蛋白質において最も高率にアミノ酸置換が確認された[12]．RC-HL株の感染性cDNAを用いて，RC-HL株のゲノムに西ヶ原株由来のG遺伝子を組み込んだキメラウイルスR（G）株が作出され，同株が西ヶ原株と同様に成熟マウスに致死的な感染を起こすことが明らかとなった[13]．この結果は，狂犬病ウイルスのG蛋白質が，さらにはその333位以外の領域が病原性に関与していることを示すものとなった．その後，西ヶ原株とRC-HL株のG蛋白質間で認められる14のアミノ酸置換のうち，242位，255位および268位の置換が両株の病原性の違いに関与することが明らかとなった[32,33]．

G蛋白質がどのような機序で病原性に関与するかについての情報が少ない中，一部の研究によってアポトーシスの関与が報告されている．Morimoto ら[18]は，成熟マウスへの皮内接種により病原性の差異が認められる2つのCVS株変異株を比較した結果，低病原性の変異株は，高病原性のそれよりも強く初代培養神経細胞にアポトーシスを誘導することを明らかにしている．低病原性変異株の感染細胞におけるG蛋白質の発現量は，高病原性のそれよりも高く，この発現量の違いが両株のアポトーシス誘導能の差異に関

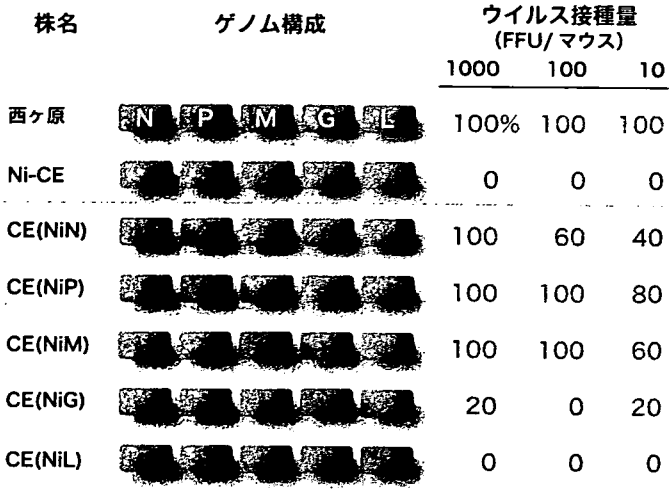

図4　各種キメラウイルスを脳内接種された成熟マウスの致死率

各種ウイルス(10-1000 FFU)を脳内接種されたddYマウス(1群5匹)を14日間観察し，各々の致死率を算出した.(文献27)

連していると考えられている．Préhaudら[21]も同様に，2株の固定毒G蛋白質のアポトーシス誘導能を比較・検討し，ウイルスの弱毒性状とアポトーシス誘導能との間の関連性を報告している．

以上，多くの研究により，狂犬病ウイルスのG蛋白質が主要な病原性決定因子であることが明らかになっている．

G蛋白質以外の狂犬病ウイルスの病原性決定因子

それでは，狂犬病ウイルスはG蛋白質以外のウイルス因子は病原性に関与しないのだろうか？

前述の西ヶ原株を鶏胚線維芽細胞で100代継代することにより，RC-HL株とは別の弱毒固定毒，Ni-CE株が確立されている（**図3**）．西ヶ原株とNi-CE株の間のアミノ酸置換の総数はわずかに15箇所であり，Ni-CE株のG蛋白質333位のアミノ酸は西ヶ原株と同じく強毒型のアルギニンであった[27]．Ni-CE株の感染性cDNAを用いて，同株のゲノムに西ヶ原株由来の遺伝子をひとつずつ導入した各種キメラウイルスを作出し，これらの病原性について解析した(**図4**)[27]．Ni-CE株のゲノムに西ヶ原株由来のN，PあるいはM遺伝子を保有するキメラウイルス（それぞれCE(NiN)株，CE(NiP)株，CE(NiM)株）は，脳内接種により成熟マウスに致死的な神経症状を引き起こした．一方，西ヶ原株由来のG遺伝子を持つキメラウイルスも成熟マウスに致死的な感染を引き起こしたが，その致死率は低く，ウイルス接種量に依存的ではなかった．この結果より，西ヶ原株とNi-CE株の病原性の違いには，主にN，PおよびM遺伝子が関連することが明らかとなった．すなわち，本研究によってG遺伝子以外のウイルス遺伝子が狂犬病ウイルスの主要な病原性決定因子となりうることが初めて示された．また，由来が同じ狂犬病ウイルス株が，その継代法の違いにより多様な機序で弱毒化されることも明らかとなった．

それでは，N，PおよびM遺伝子はどのような機序で病原性に関連するのだろうか？　感染初期（脳内接種後2～3日）の成熟マウス脳内におけるNi-CE株の増殖性は，西ヶ原株のそれに比べて顕著に低いことが分かっている（未発表）．このことから，両株の病原性の違いに宿主の自然免疫に対する抵抗性の差が関与することが示唆された．最近，狂犬病ウイルスのP蛋白質が宿主細胞の転写因子であるSTAT1の核内移行を阻害することにより，I型およびII型インターフェロン（IFN）シグナル経路をブロックすることが報告されている[36]．そこで，西ヶ原株，Ni-CE株およびキメラウイルスCE（NiP）株のI型IFN抵抗性に差異があるか否かについて検討を行ったところ，強毒の西ヶ原株およびCE（NiP）株のI型IFNに対する抵抗性は，弱毒のNi-CE株のそれよりも強いことが明らかにされた(**図5**)[28]．その後の解析により，西ヶ原株P蛋白質のIFNシグナル阻害能がNi-CE株P蛋白質のそれよりも強いことも分かってきた（未発表）．一方，NおよびM遺伝子がどのような機序で病原性に関連するのかついては未だ明らかにされておらず，今後の解明が待たれる．

安全な弱毒生ワクチンの開発に向けて

上記のように，狂犬病ウイルスG蛋白質以外のウイルス因子が病原性に関与することが明らかとなった．この知見は安全な弱毒生ワクチンを開発する上でも重要である．これまで，野生動物の経口免疫を目的に，G蛋白質333位のアミノ酸を弱毒型に置換したウイルスが弱毒生ワクチンとして用いられてきた[1, 2, 9, 16, 24]．ヨーロッパを中心とした各国では，この弱毒生ワクチンを混入させた餌を野外に散

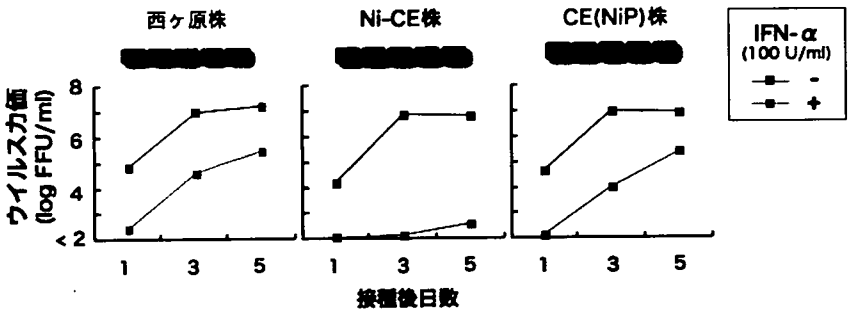

図5 IFN-α存在下における各ウイルスの増殖曲線

各ウイルスをマウス神経芽腫由来NA細胞にmoi=0.01で接種後，100 U/mlのIFN-αの存在下あるいは非存在下でウイルスを培養した．接種1，3および5日後に回収した培養上清中のウイルスの力価（FFU/ml）を測定した．なお，予備試験によって，NA細胞のIFN産生系は機能的に欠落していることが確認されている．（文献28の図を改変）

布することにより，キツネなどの野生動物における狂犬病の発生数を顕著に減少させている．しかし，この弱毒変異ウイルスのG蛋白質333位のアミノ酸が強毒型のアルギニンあるいはリジンに置換した場合，ウイルスの病原性は強毒化することが報告されている[16]．

既に述べたように，Ni-CE株の弱毒化には主にN，PおよびM遺伝子が関連している．そこで，G蛋白質333位のアルギニンを弱毒型のアミノ酸に置換した同株の変異株を作製すれば，複数の機序で弱毒化したウイルス株を得ることができる．このようなウイルス株は，理論的に，上に述べた現行の弱毒生ワクチン株よりも病原性が復帰しにくいと考えることができる．今後，感染性cDNAを用いて複数の機序により弱毒化したワクチン株を作出することにより，より安全性の高い弱毒生狂犬病ワクチンの開発を期待することができる．

狂犬病ウイルスの病原性に関する最近の知見

最近，脳内における狂犬病ウイルスが排除されるか否かを決定する要因として，血液-脳関門の透過性が重要であることが報告されている[22]．すなわち，弱毒の固定毒に感染したマウスの血液-脳関門の透過性が，街上毒感染マウスのそれよりも顕著に高いことが明らかとなった．その結果，弱毒固定毒感染マウスでは，脳組織への免疫エフェクター細胞の浸潤が可能になり，ウイルスが脳から排除されると考えられている．また，固定毒感染マウスにおける血液-脳関門の透過性上昇の機序に，脳血管周囲に浸潤した$CD4_+$ T細胞が関与することも報告されている[20]．これらの$CD4_+$ T細胞に由来するIFN-γが血管内皮細胞に作用してペルオキシニトライト・ラジカル（ONOO-）を産生させ，結果的に血液-脳関門の透過性を上昇させることが示された．

以上の報告は，これまでの狂犬病ウイルスの病原性に関する研究の流れに，新たな方向性を示すものと考えられる．それと同時に，血液-脳関門が狂犬病の治療法の標的となる可能性を示した．すなわち，狂犬病患者の血液-脳関門の透過性を上昇させることができる薬剤が開発されれば，有力な本病の治療薬となるかもしれない．実際，暴露後免疫を受けずに狂犬病を発症した患者が回復するという稀なケースが最近報告されたが，この患者の血液-脳関門の透過性が上昇していたことを示唆するデータが得られている[5, 37]．以上のように，狂犬病ウイルスの病原性の違いが生じる機序を解明し，その情報を蓄積することは，狂犬病の治療法を確立する上で極めて重要と言える．

最後に

上に述べたように，これまでの狂犬病ウイルスの病原性に関する研究は，ウイルスの病原性決定因子の同定を中心に行われてきた．今後の研究は，決定された病原性決定因子がウイルス-宿主相互作用にどのような影響を与えるのかという命題を中心に展開されていくことが予想される．このような情報の蓄積が，安全性の高い新規弱毒生ワクチンの開発や治療法の確立につながっていくことを期待したい．

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Progression in studies on pathogenesis of rabies virus

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Rabies virus causes lethal neurological symptoms in humans and animals. Rabies epidemics have continued to occur throughout the world, despite the fact that rabies can be effectively prevented by vaccination. The development of inexpensive and safe attenuated live vaccines and the establishment of cures are the keys to control rabies. To achieve these objectives, it is important to elucidate mechanism by which rabies virus causes disease. Here, previous studies on the pathogenesis of rabies virus are reviewed and ways to apply previous findings to rabies control are also discussed.

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Control of rabies: Epidemiology of rabies in Asia and development of new-generation vaccines for rabies

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Abstract

Rabies is an enzootic viral disease widespread throughout the world. Although it is a vaccine-preventable disease, the annual number of human deaths caused by rabies is estimated to be 32,000 in Asia. Phylogenetic analysis based on sequence data of the partial N gene of rabies viruses in Asia has shown that the viruses are divided into five genogroups, distributed in Middle East, South Asia, South East Asia, Malay, and Arctic regions. The genetic relationships among these rabies viruses agree basically with the results of previous studies. Meanwhile, new types of vaccines are being developed by applying gene manipulation techniques to rabies virus in order to overcome the disadvantages of current vaccines. This article reviews the molecular epidemiology of rabies in Asia and progress made in the development of new-generation rabies vaccines with the goal of elimination or control of rabies in Asia.

Keywords: Rabies virus; Molecular epidemiology; Asia; N gene; Vaccine; Reverse genetics; M gene

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Résumé

La rage est une maladie virale enzootique très répandue dans le monde entier.

Bien qu'il s'agisse d'une maladie évitable par vaccination, le nombre de décès imputé à la rage est estimé à 32,000 en Asie. Une analyse de la phylogénèse basée sur les données séquentielles du gène partiel N des virus de la rage en Asie a montré que les virus sont divisés en cinq génogroupes, répartis dans les régions du Moyen-Orient, de l'Asie du Sud, de l'Asie du Sud-Est, de la Malaisie et de l'Arctique. Les relations génétiques entre ces virus de la rage s'accordent fondamentalement avec les résultats des études précédentes. En attendant, des nouveaux types de vaccines sont développés en utilisant des techniques de manipulations génétiques au virus de la rage de façon à surmonter les désavantages des vaccins actuels. Cet article examine l'épidémiologie moléculaire de la rage en Asie et les progrès effectués dans le développement de vaccins contre la rage de nouvelle génération dans le but d'éliminer ou de contrôler la rage en Asie.

Mots clés: Virus de la rage; épidémiologie moléculaire; Asie, Gène N; Vaccin; génétique réverse; gène M

1. Introduction

Rabies is an enzootic viral disease widespread throughout the world. It is transmitted to other animals and humans from infected domestic and wild animals. Once neurologic symptoms of the disease develop, rabies is fatal to both animals and humans. The annual number of human deaths worldwide caused by rabies is estimated to be 55,000 [1], mostly in rural areas of Africa and Asia. An estimated 10 million people receive post-exposure treatments each year after being exposed to rabies-suspect animals.

In Asia, rabies is one of the most important diseases because of the high human mortality rate and high costs of prevention and treatment. A survey has shown that Asia carries a larger part of the public health burden of rabies with an estimated 32,000 deaths and 96.5% of the economic burden of rabies in the developing world with US$ 560 million spent each year mostly on post-exposure prophylaxis [1]. At present, only a few countries, including Japan, Singapore, and Taiwan, are free of rabies. It has been reported that rabies spread from neighboring infected areas to rabies-free areas, the border region of South Korea and Flores island of Indonesia, in 1993 and 1997, respectively [2,3]. Attention must therefore be given to this disease as a potential emerging or re-emerging infectious disease even in rabies-free countries.

Rabies virus belongs to the genus *Lyssavirus* of the family *Rabdoviridae*. The genus is composed of rabies virus (genotype 1) and rabies-related viruses, including Lagos bat (genotype 2), Mokola virus (genotype 3), Duvenhage virus (genotype 4), European bat lyssaviruses 1 and 2 (genotypes 5 and 6, respectively), and Australian bat lyssavirus (genotype 7) [4]. The virus has a negative-sense single-stranded RNA genome of approximately 12 kb containing coding information for nucleocapsid (N), phosphoprotein (P), matrixprotein, glycoprotein (G), and RNA polymerase (L).

Although it is difficult to distinguish field isolates of rabies virus serologically because of a single serotype, rabies isolates can be distinguished by genetic analysis.

The development in recent years of molecular methods of analysis such as reverse transcription and polymerase chain reaction (RT-PCR) and techniques for sequencing rabies genes has led to a better understanding of the distribution and genetic characteristics of rabies virus at both the global level [5,6] and regional levels, such as in Asia [7–16]. The establishment of a database of rabies virus genes from molecular epidemiological analysis has enabled more precise definition of virus type and will be useful for tracing transmission of rabies virus from infected animals to humans or to other animals. Earlier, restriction fragment polymorphism analysis using PCR-amplified products actually enabled the origin of rabies virus infection among immigrants to the United States to be traced [17]. It is also important to establish a strategic plan for rabies prevention and control based on an understanding of the ecology and dynamics of rabies viruses in nature [15].

Humans become infected with rabies by the bite of an infected animal, mostly a rabid dog in Asia and Africa. Therefore, it is necessary to control rabid dogs by mass vaccination of dogs in epidemic areas for elimination of rabies. Development of fatal disease in humans bitten by infected animals can also be prevented by post-exposure vaccination. These indicate that rabies is a vaccine-preventable disease. However, many people are still becoming victims of the disease in the present situation, which is far from a situation of elimination or control of rabies in Asia and Africa as described above, despite the fact that safe and effective modern vaccines exist for both human and veterinary use. Since modern inactivated vaccines derived from tissue culture are too expensive to be used for immunization and treatment of humans or animals in developing countries, vaccines from nervous tissues of rabies-infected animals are still produced and used in some Asian countries. The premodern vaccines are less effective, require repeated visits to the hospital, and often cause severe side-effects [18,19]. To overcome these disadvantages of current vaccines, the development of new types of vaccines by applying gene manipulation techniques to rabies virus is in progress [20–23].

In this article, the molecular epidemiology of rabies in Asia and progress made in the development of new-generation rabies vaccines with the goal of elimination or control of rabies in Asia are reviewed.

2. Molecular epidemiology of rabies in Asia

Smith et al. [5] analyzed a 200-b region of the N genes from 87 rabies virus isolates from various parts of the world and showed for the first time that six unique genetic groups exist globally and that four of these groups are distributed in Asia. Interestingly, these data suggested that a historical reconstruction of events leading to the introduction of rabies into an area would be possible. From analysis of the complete nucleoprotein (N)-coding genes of 69 isolates from various parts of the world, it was also shown that at least 11 phylogenetic lineages could be identified in accordance with their geographical localization and species of origin [6].

Many genetic data on rabies virus, especially data on the N gene, have been deposited in a database. Phylogenetic analysis was therefore performed by using the sequence data of the partial 240 b corresponding to nucleotides at positions 39–278 of the N gene (open reading frame) of representative rabies viruses in Asia and its vicinity with Australian bat lyssavirus as an outgroup (Fig. 1). The results indicate that rabies viruses in Asia are divided into five genogroups, distributed in Middle East, South Asia, South East Asia, Malay, and Arctic regions (Fig. 2). These results are slightly different from those in the earlier analysis by Smith et al. The genetic relationships among these rabies viruses agree basically with the results of previous analyses at a regional level in Asia [7–16].

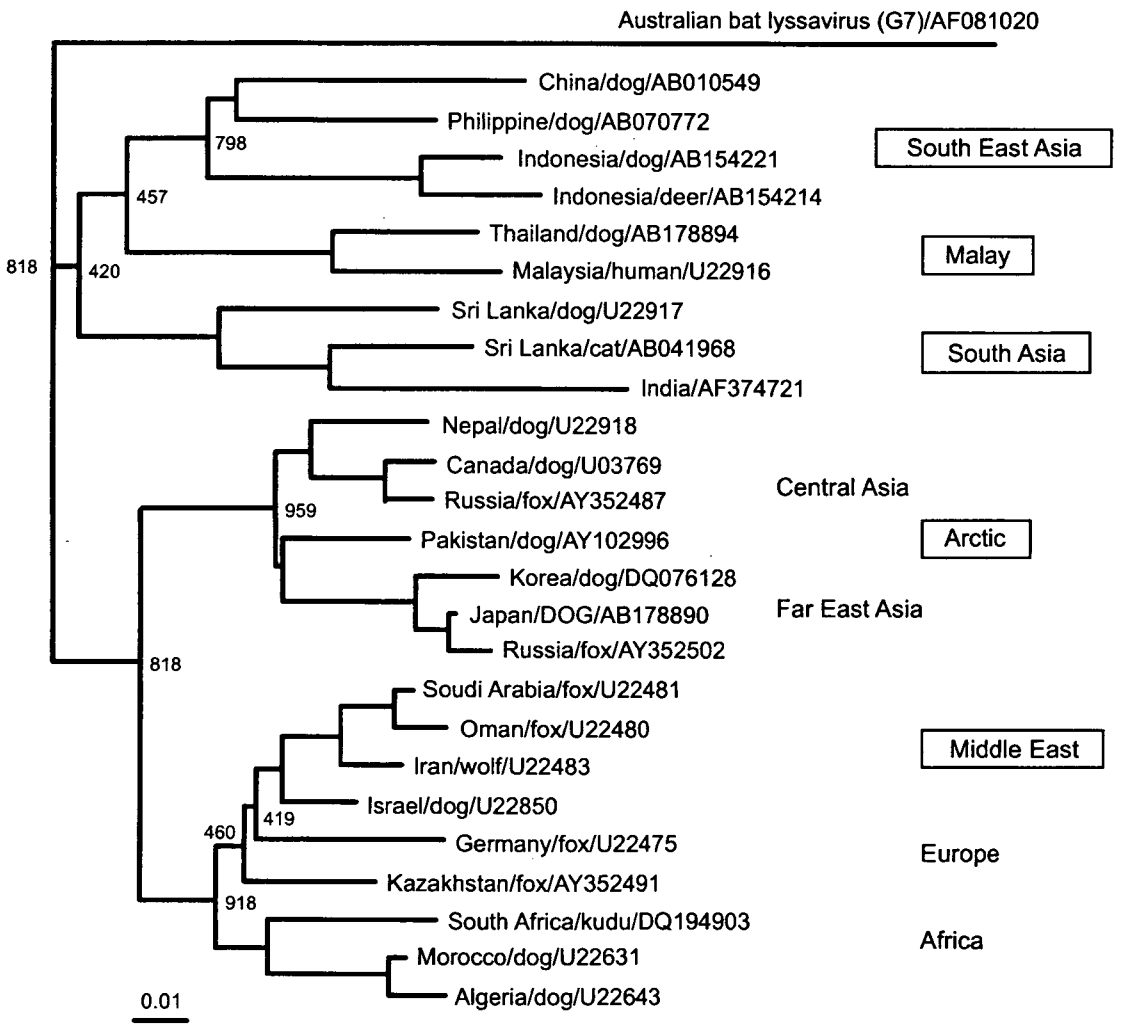

Fig. 1. A phylogenetic tree based on the nucleotide sequences of 240 b corresponding to the nucleotides at positions 39–278 of the N gene (open reading frame) of representative rabies viruses in Asia and its vicinity with Australian bat lyssavirus as an outgroup. Sequences were analyzed by the neighbor-joining method using Clustal X. Numbers at nodes are bootstrap probabilities calculated using 1000 replicates.

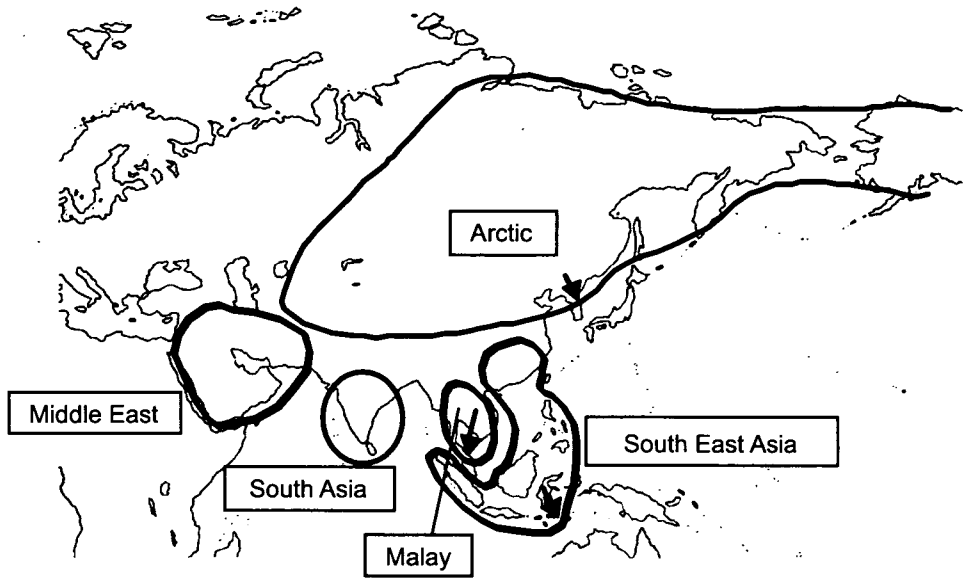

Fig. 2. Geographical distribution and dynamics of rabies viruses in Asia. Genogroups were determined by phylogenetic analysis based on nucleotide sequences of the partial N gene. Arrows show the migration of rabies viruses.

2.1. Arctic (-like) rabies viruses

Kuzmin et al. [13] analyzed the N genes of 55 rabies virus isolates originating from different regions of the former Soviet Union and compared with them with N genes of isolates from various parts of the world. The isolates were divided to two clusters, clusters I and II, with two distinct groups and three distinct groups in clusters I and II, respectively. The isolates in the former genogroup belong to Arctic (-like) viruses originating from Eurasia and North America [24]. Arctic and Arctic-like rabies viruses are located in the polar circle and the border area with Mongol and in the border area with China and Far East Russia, respectively. Their analyses also showed that these Arctic viruses circulated mainly in wild animals: Arctic fox in the Arctic area and raccoon dog in the Far Eastern area.

Kissi et al. [6] reported that an isolate from Nepal was grouped in the Arctic viruses. As shown in Fig. 1, this is supported by our phylogenetic analysis using partial N genes of rabies viruses in Asia. It has also been reported that two isolates from the northeastern area in Iran were shown by phylogenetic analyses based on the nucleotide sequence of the P gene to be closely related to Arctic viruses [12].

In South Korea, rabies re-emerged in 1993, although the disease had been eradicated in 1985 (Fig. 2). It was reported that isolates in the current outbreak of rabies are closely related to Arctic viruses on the basis of results of phylogenetic analyses [14]. The fact that the raccoon dog is the main epidemic carrier of rabies in Korea and the results of these studies supported the conclusion of the study in the former Soviet Union [13] that raccoon dogs take part in the circulation of rabies virus within their natural territories in the Far East. Interestingly, an isolate from a dog in Japan in the 1940s, when rabies was still endemic, was also closely related to

isolates in the Far Eastern area of Russia and Korea, indicating that it belongs to Arctic-like viruses (Fig. 1) [7].

2.2. Rabies viruses in the Middle East

As shown in Fig. 1, rabies viruses in the Middle East form one genogroup. These viruses are related more closely to viruses distributed in Europe and Africa rather than those in Asia. This is supported by the results of phylogenetic analysis based on the partial N gene in a previous study showing that many isolates from Israel were included in the same genetic group of isolates from Middle-Eastern countries, South Lebanon, Iran, Oman, and Saudi Arabia [8]. It was also shown that the isolates throughout Iran belonged to the same lineage disseminated widely in the Middle East, Europe, Africa, and America except for two isolates from the northeastern area, which belonged to the Arctic group on the basis of results of genetic analysis of the partial P gene sequences as described above.

2.3. Rabies viruses in South Asia

Rabies viruses from Sri Lanka and India form one lineage independently based on the partial N gene sequence (Fig. 1). It was shown by comparing partial sequences of the N gene that the isolates from Sri Lanka formed a specific cluster that included an isolate from India [9]. Recently, Nagarajan et al. [16] showed by analyses of the cytoplasmic domain of the G gene and the G-L intergenic region that the isolates from India were included in one lineage.

2.4. Rabies viruses in South East Asia

In an earlier study, it was shown that rabies viruses from Philippines formed one of six genetic groups uniquely based on partial sequences of the N gene [5]. It has also been reported that many isolates from Philippines belonged to a different lineage from other Asian isolates [10]. As shown in Fig. 1, phylogenetic analysis of the partial N gene shows that an isolate from the southern part of China [7] is included in the same genogroup as that of Philippines isolates. We have recently analyzed N genes of isolates from Indonesia and have obtained results showing that those isolates formed one lineage and were closely related genetically to the isolates from Philippines and China (Fig. 1). Rabies was introduced to Flores Island from Sulawesi Island by sailors in 1997 (Fig. 2), and its outbreak resulted in at least 113 human deaths from 1998 to 2002 [3]. The isolates from both islands were included in the same genetic lineage (submitted for publication).

2.5. Rabies viruses in Malay Peninsula

We revealed that isolates from Thailand belonged to the same genogroup consisting of two genetic clusters and were genetically different from isolates from other Asian areas [7,11]. Based on results of detailed genetic analyses of these isolates

and on the historical background of Thailand, we hypothesize that one of rabies viruses that were prevalent in central and northeastern areas might have been introduced to the lower southern parts of Thailand (Fig. 2). This hypothesis is supported by recent results of more detailed analyses of N genes of rabies virus in Thailand [15]. The present phylogenetic analysis also shows that this genogroup includes an isolate from Malaya [6], although it was collected from an unknown part of Malay Peninsula.

In conclusion, the analyses show that there are at least five genetic groups of rabies viruses distributed in Asia. Rabies viruses in four of the five groups are prevalent locally and those in the remaining group, the Arctic (-like) rabies viruses, are distributed widely in the Northern Hemisphere from Canada to the northeastern area of the Middle East (Fig. 2). Further genetic analyses of rabies viruses, especially in countries and areas with little genetic information on rabies, such as Iraq, Afghanistan, Bhutan, Bangladesh, Myanmar, Laos, Cambodia, Vietnam, and China, are needed to elucidate the dynamics of rabies in Asia.

3. Development of new-generation rabies vaccines by gene manipulation of rabies virus

3.1. Current rabies vaccines

Currently, inactivated rabies vaccines are most popularly being used for prevention of rabies around the world. However, inactivated vaccines, especially vaccines derived from tissue culture, are too expensive for vaccination of people and animals in developing countries. The high production cost of the vaccines is mainly due to the requirement of large amounts of viral antigen to sufficiently induce a protective immune response in the inoculated animal. On the other hand, inactivated vaccines from nerve tissues of rabies virus-infected animals (e.g., Semple rabies vaccine) can be produced at a lower cost. However, such vaccines can cause serious side-effects such as autoimmune encephalomyelitis in inoculated animals [18,19]. Furthermore, such vaccines require a needle-tipped syringe for delivery, hindering vaccination in developing countries, where a shortage of syringes and needles has continuously been a serious problem.

Attenuated live vaccines, on the other hand, efficiently elicit a protective immune response with a smaller amount of the virus, because the vaccine virus propagates and synthesizes viral antigen in the inoculated animal. These vaccines can generally be produced at a lower cost than inactivated vaccines and can be delivered by needle-free methods such as oral inoculation [25–27]. However, attenuated live vaccines have a serious problem: the vaccine virus sometimes causes rabies in the inoculated animal [28,29] by its residual virulence or pathogenic mutation during viral propagation in the body. This is the main reason for generally using inactivated rabies vaccines rather than attenuated live vaccines. Also, development of attenuated live vaccines needs much time because the vaccine virus is generally established by serial passages of the parental virus in cultured cells. During the passages, the virus acquires mutations on its genome, which are related to

its adaptation to the cultured cells and some of which are coincidentally associated with its attenuation.

3.2. Gene manipulation of rabies virus for vaccine development

The problems of the current rabies vaccines described above indicate the type of new-generation rabies vaccines that are needed. Attenuated live rabies vaccines that are safer and more effective than the currently available vaccines are required for prevention of rabies in the world, especially in developing countries. In order to accomplish this purpose, a gene manipulation system (also called a reverse genetics system or infectious cDNA) of rabies virus has recently been utilized in many studies.

Fig. 3 shows the principle of the gene manipulation system of rabies virus, which was first established in the SAD B19 strain by Schnell et al. [30] in 1994. Using almost the same principle, systems for the other five strains have been reported [31–35]. This system can be rephrased as "a method for recovering infectious virus from cloned cDNA". Briefly, a plasmid expressing full-length anti-genomic RNA (genome plasmid) and three plasmids expressing N, P, and L proteins of the virus (helper plasmids) are transfected into a cell. Afterward, the anti-genomic RNA and the proteins form an anti-genomic ribonucleoprotein (RNP) complex. Since the anti-genomic RNP complex has the same biological activity as that generated in rabies virus-infected cells, genomic RNA is synthesized using this anti-genomic RNP as a template, followed by synthesis of mRNA from the genomic RNP and expression of viral protein. Assembly of the genomic RNP and the other viral proteins, M and G proteins, results in generation of an infectious recombinant rabies virus.

To genetically manipulate the rabies virus, it is only necessary to change the viral cDNA sequence on the genome plasmid by a conventional molecular cloning method and to transfect the modified genome plasmid, together with helper plasmids, into a cell in the same manner as that described above. Using this gene

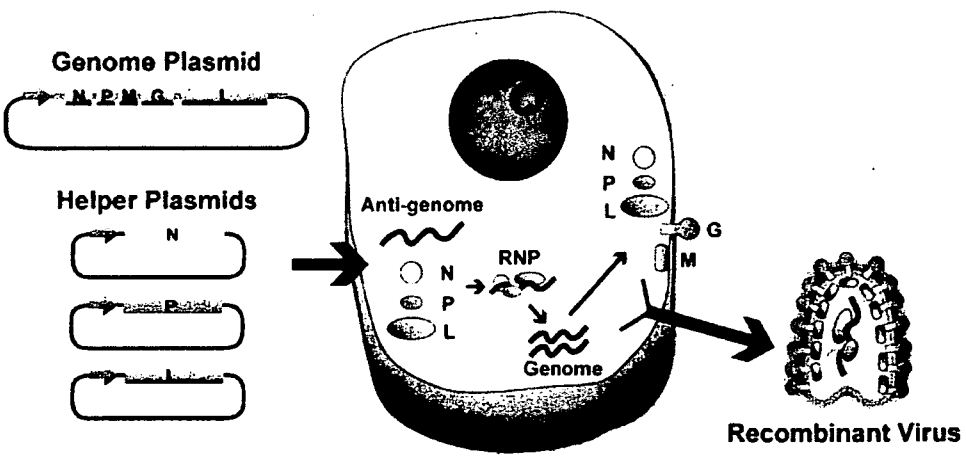

Fig. 3. Principle of the gene manipulation system of rabies virus.

manipulation system of rabies virus, an attenuated live vaccine virus can be established much more quickly than that using the classical method by cell culture passages of the virus. Also, in this system, it is possible to discretionarily change biological characters of the vaccine virus in order to increase its safety and immunogenicity, whereas the classical method relies on attenuation-related mutations coincidentally occurring during the passages.

3.3. New-generation rabies vaccines

The candidates for attenuated live vaccines generated by gene manipulation can be distinguished into the four categories. First, insertion of a foreign gene into the viral genome, which causes attenuation of the virus and effective immune response, was utilized for generation of attenuated vaccine viruses. Pulmanausahakul et al. [36] generated a recombinant rabies virus expressing a proapoptotic protein, cytochrome *c* (SPBN-Cyto *c*(+) strain), and showed that the SPBN-Cyto *c*(+) strain strongly induced apoptosis in infected cells and, consequently, was more attenuated than the negative control virus carrying inactivated cytochrome *c* gene (SPBN-Cyto *c*(−) strain). The SPBN-Cyto *c*(+) strain also induced a protective immune response in inoculated mice more effectively than did the SPBN-Cyto *c*(−) strain. Very recently, the same group reported that a recombinant virus expressing tumor necrosis factor alpha was also attenuated and strongly induced inflammation in the brains of infected mice [37].

Second, it has also been reported that an attenuation-related mutation was introduced into the viral genome to obtain an attenuated virus. Since an arginine or lysine residue at position 333 in the G protein is well known as a pathogenic determinant [38–40], this residue was chosen as a target to generate the attenuated virus. For example, Morimoto et al. [20] constructed many recombinant viruses harboring the G gene from various rabies virus strains with/without a mutation at the position and showed that alteration of the amino acid residue is useful for generation of the attenuated virus. On the other hand, Mebatsion [41] reported that deletion of the dynein light-chain binding site in P protein, which is thought to be important for axonal transport of the virus, reduces peripheral infectivity of the virus, as shown by intramuscular inoculation of the mutant virus into suckling mice.

Third, in order to enhance immunogenicity of the attenuated vaccine virus, an attempt has been made to increase the expression level of G protein in infected cells, the most important viral antigen to elicit protective immunity, by insertion of an additional G gene into the genome, as reported by Faber et al. [42]. In that study, a recombinant rabies virus (SPBNGA-GA strain) carrying two identical G genes was generated. Both of the G genes contained the attenuation-related mutation with a change in the amino acid residue at position 333 in the G protein as described above. Faber et al. showed that cultured cells infected with the SPBNGA-GA strain produced twice as much G protein as did cells infected with the virus carrying only a single G gene (SPBNGA strain) and that the strain induced apoptosis more strongly. They also showed that immunization of mice with the SPBNGA-GA strain resulted in more efficient protective immunity than that with the SPBNGA strain.