events should be well-established, and surveillance for bioterrorism incidents should be integrated into the primary disease surveillance systems. A multilevel laboratory response network, linking clinical laboratories to public health agencies and transferring diagnostic technologies to local levels, can facilitate rapid and accurate diagnosis and characterization of the etiologic agent. The response protocol should include details of the epidemiologic investigation designating teams to investigate unexplained or suspicious illness, medical treatment and prophylaxis including the availability and accessibility of vaccines and antibiotics, and environmental decontamination. As public health professionals, effective communication with both the public and other health care and public health personnel is essential. Provision of accurate and timely updates through the news media and the provision of a consistent, reliable means to access emergency information via a hotline or the internet can help limit panic and disruption of daily life among the public. Coordination of activities among health personnel, and establishing mechanisms to ensure rapid notification and exchange of information can be most useful in effectively managing a bioterrorist incident.

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Figure 1. Bioterrorism Agent Categorization

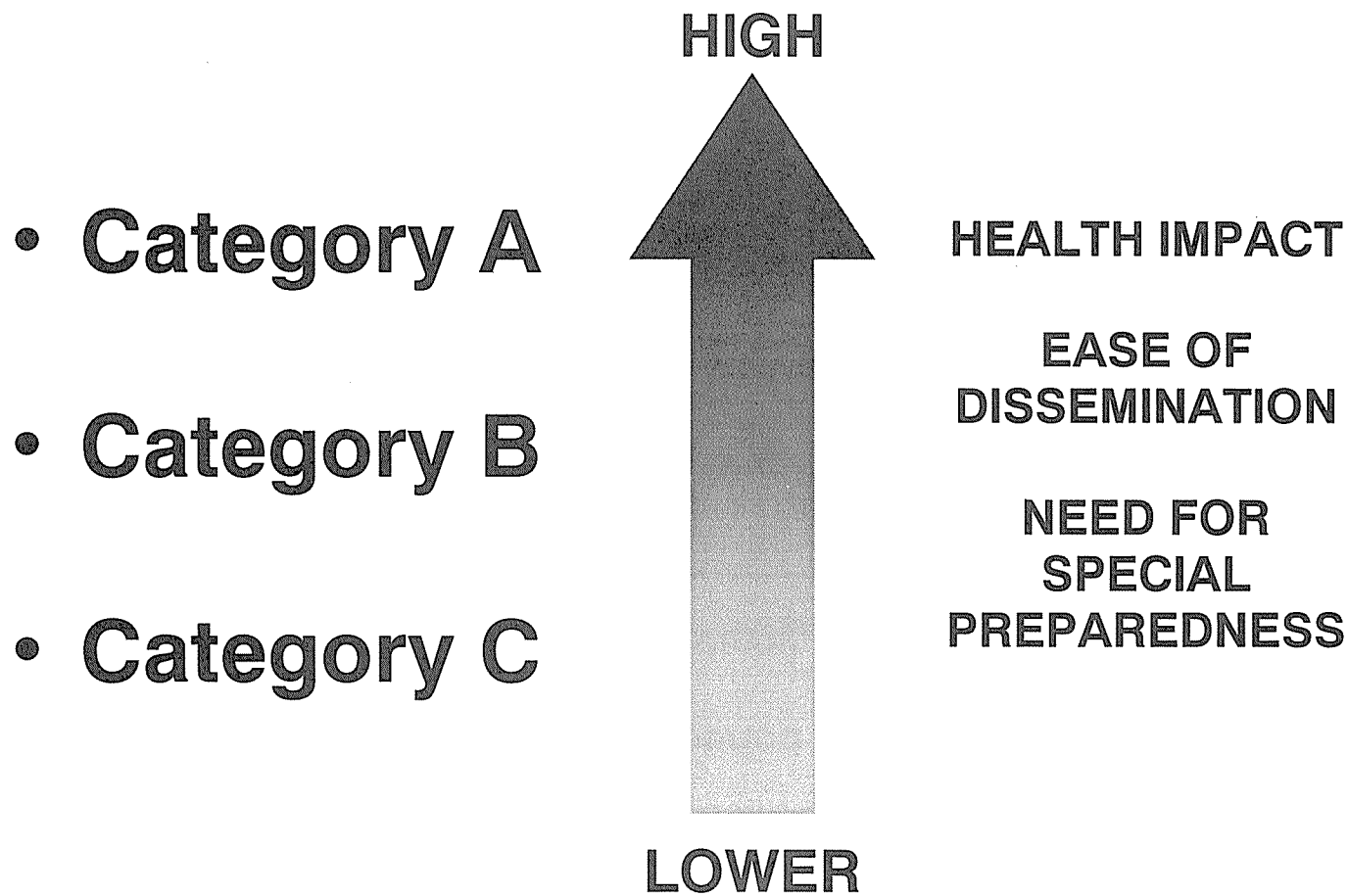

Table 1. Overview of Category A Bioterrorism Agents.

Agent	Spread Person to person?	Infective dose	Incubation period (days)	Vaccine Availabl e?	Persistence in the Environment
Anthrax	No	<10,000	1-7	+	Very stable
Botulism	No	0.001 (μg/kg)	12-36 hours	+/-	Stable
Plague	High	<500	1-6	-	Stable
Smallpox	Yes	<100	2-14	+	Very stable
Tularemia	No	<50	3-5	+	Stable
VHF	Moderate	<10	2-21	-	Unstable

μg, micrograms
kg, kilogram (of body weight)
VHF, Viral hemorrhagic fevers

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Special Issue

Commentary

Strategic Approach to Information Security and Assurance in Health Research

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Abstract

Information security and assurance are an increasingly critical issue in health research. Whether health research be in genetics, new drugs, disease outbreaks, biochemistry, or effects of radiation, it deals with information that is highly sensitive and which could be targeted by rogue individuals or groups, corporations, national intelligence agencies, or terrorists, looking for financial, social, or political gains. The advents of the Internet and advances in recent information technologies have also dramatically increased opportunities for attackers to exploit sensitive and valuable information.

Government agencies have deployed legislative measures to protect the privacy of health information and developed information security guidelines for epidemiological studies. However, risks are grossly underestimated and little effort has been made to strategically and comprehensively protect health research information by institutions, governments and international communities.

There is a need to enforce a set of proactive measures to protect health research information locally and globally. Such measures should be deployed at all levels but will be successful only if research communities collaborate actively, governments enforce appropriate legislative measures at national level, and the international community develops quality standards, concluding treaties if necessary, at the global level.

Proactive measures for the best information security and assurance would be achieved through rigorous management process with a cycle of "plan, do, check, and act". Each health research entity, such as hospitals, universities, institutions, or laboratories, should implement this cycle and establish an authoritative security and assurance organization, program and plan coordinated by a designated *Chief Security Officer* who will ensure implementation of the above process, putting appropriate security controls in place, with key focus areas such as *policies and best practices*, *enforcement and certification*, *risk assessment and audit*, *monitoring and incident response*, *awareness and training*, and *modern protection method and architecture*. Governments should enforce a comprehensive scheme, and international health research communities should adopt standardized innovative methods and approaches.

Key words: security and assurance, health research information, proactive measures, ISMS, CSO/CISO

Risks involved in health research information

Information security and assurance is an increasingly critical issue in health research. Health research deals with information that is highly sensitive, be it health care record of individuals/populations, genetic epidemiology, disease outbreak information of nations, or data on new drugs/bio-chemicals. They are targets for rogue individuals or groups, corporations, national intelligence agencies, or terrorists, looking for financial, social, or political gains. Insurance companies are eager to discover detailed medical histories of their customers and their customers' families to define the most cost effective insurance premiums. Corporations could recruit new staff, decide assignments, or select future executives, based on genetic profiles of

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employees. Early disease outbreak news is extremely valuable to stock exchange traders and speculators, whereas terrorists and intelligence agencies may have political reasons to interfere with early outbreak alert and response operations. Alternatively, a mere unwarranted disclosure of outbreak information could have a profound impact on the economy of nations who depend on tourism. Web sites posted by health scientists describing the impact of new deadly bio-chemical or radiation materials could be a textbook for terrorists.

The advent of the Internet and advances in recent information technologies have revolutionalized the way health research is conducted, and have made it extremely efficient to collect, store, exchange and process vast amounts of scientific information, yet have dramatically increased opportunities for attackers to exploit sensitive and valuable information to their ends through sophisticated but rogue technological means. To make matters worse, research scientists tend to pay little attention to the security of their data. Laboratory systems are much less well protected than operation systems.

Current countermeasures and their problems

Some government agencies have deployed legislative measures to protect the privacy of health information, especially in the health care sector, and developed standard information security guidelines for epidemiological studies. However, the risks are grossly underestimated and little effort has been made to strategically and comprehensively protect the health research information of universities, hospitals, institutions, government agencies, and international communities, through adequate security management processes. There are hardly any health research centers in the world today, except those dealing with highly confidential military intelligence or counter-terrorism health data, for example, where an authoritative information security program has been established and implemented. Not to mention that these centers lacks institutionalized information security risk assessment processes. They have, simply, no idea what critical assets are there to protect, from who and why, when it comes to health research information.

There is a need to promote and enforce a set of proactive measures to strategically and comprehensively protect health research information both locally and globally. Such measures should be deployed at all levels, but will be successful only if research communities collaborate actively, supporting governments enforce legislative measures at a national level, and international community develops quality standards, concluding treaties if necessary, at the global level. International collaboration is necessary particularly to address security issues involved in unprecedented free flows of, and easy access to, scientific information across the Internet.

Strategic approach

The best proactive measure would be a rigorous security management process where a cycle of "plan, do, check, and act" is enforced (Fig. 1). The approach described is based on the British Standard Institute's BS7799-2:2002 (1), to be superseded by the International Standard Organization's ISO/

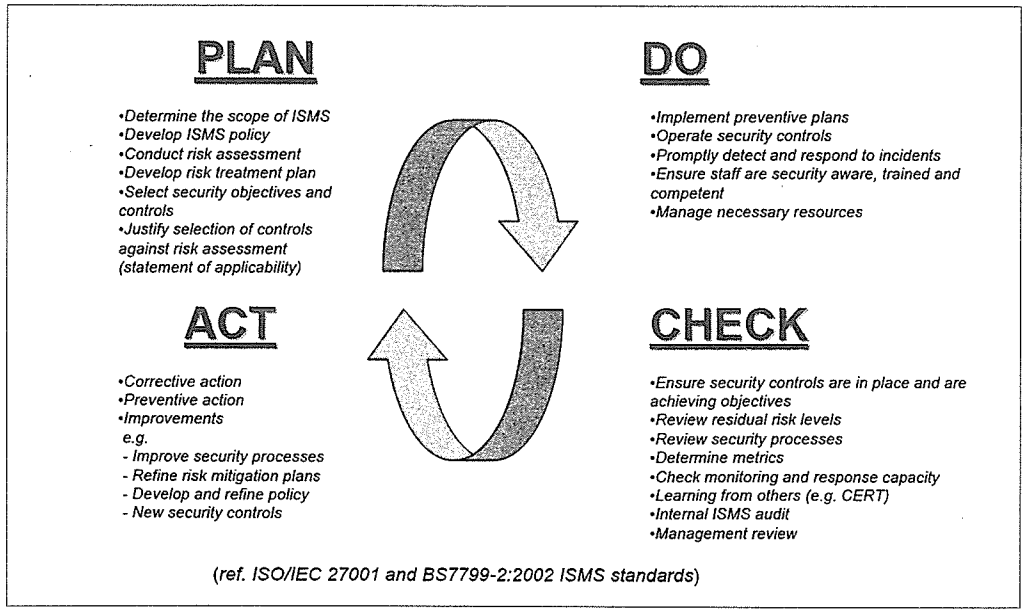

Fig. 1 Information Security Management System (ISMS) cycle.
Note: Information Security Controls and Best Practices are categorized by ISO/IEC17799:2005 (3) as: 1) security policy, 2) organization of information security, 3) asset management, 4) human resources security, 5) physical and environmental security, 6) communications and operations management, 7) access control, 8) information systems acquisition, development and maintenance, 9) information security incident management, 10) business continuity management, and 11) compliance.
Security control measures would include: 1) governance, technical and end-user policies; standards and reference architecture, 2) auditing and compliance assessment, certification, 3) vulnerability management, anti-malware (virus, worms, Trojan horses, SPAMS, spyware) systems, 4) access-control enforcement (firewalls/IPS (intrusion prevention systems), authentication, authorization, accounting systems, etc.), 5) monitoring, surveillance and response, incident response teams, and 6) awareness and training programs.

IEC27001. (ISO/IEC27001 for Information Security Management Systems (ISMS), could be considered one of the key quality assurance standards along with ISO9001 for Quality Management Systems (QMS), ISO14001 for Environment Management Systems (EMS), and OHSAS18001 for Health and Safety Management Systems (HSMS).)

The ISMS cycle consists of: the PLAN phase, where the ISMS's scope is defined, the ISMS's policy is developed, risk assessment is conducted, a risk management/risk treatment strategy is determined, security objectives and controls are selected, and selected controls are justified against risk assessment (i.e. statement of applicability (SOA)); the DO phase, where preventive plans are implemented, security controls are actually operated, and security incidents are promptly detected and responded to; the CHECK phase, where checks are made to ensure that security controls are firmly in place and are achieving goals, residual risk levels are reviewed, security processes are reviewed, metrics for evaluation are determined, monitoring and response capacity is checked, learning from others, such as CERT/CC (2), is done, an ISMS audit is conducted, and a management review is executed; and the ACT phase, where actions are taken to correct, prevent and improve (e.g. improvement of security processes, refinement of risk mitigation plans, development of new policies and refinement of existing policies, and design and implementation of new security controls).

Each health research entity, such as hospitals, universities, institutions, or laboratory centers, should implement this ISMS cycle, and establish an authoritative security and assurance management organization. Such an organization should be headed by a *Chief Security Officer* (CSO), or a *Chief Information Security Officer* (CISO), who takes charge of all information security and assurance issues and develops a security plan, coordinating the security program, ensuring the implementation of ISMS processes and manage/coordinates appropriate security controls, with key focus areas such as: *policies and best practices*, *enforcement and certification*, *risk assessment and audit*, *monitoring and incident response*, *awareness and training*, *and modern protection methods and architecture* (4). These six areas are particularly important because:

Policy and best practices: Policy describes exact rules and steps to be followed in order to improve security, whereas best practices are the behaviors which are considered to be effective by most industries, the public and experts, and followed often without formal assessment. Since security is not an exact science, both are needed.

Enforcement and certification: Policies and best practices are not effective unless they are enforced. Certification is to accredit officially and authoritatively compliance to policies, and is one of most effective methods of enforcement.

Risk assessment and audit: Risk is a multitude of [asset value]×[threat likelihood]×[threat impact]×[vulnerability], where critical assets could be tangible assets such as infrastructure—hardware and software, people, data, knowledge and services, or intangible assets such as privacy, reputation, credibility and absence of legal liability. Risks are moving targets, which change in time. Risk assessment is a key to understanding the current state of security at an organization, and should be conducted regularly. Audit verifies the successful implementation of security controls.

Monitoring and incident response: In security, prevention, detection, and response are all necessary. Most information security is preventive in nature, which is a countermeasure to provide two things: a) a barrier to overcome and b) time to overcome the barrier. Without detection and response, however, the preventive countermeasure is much less effective. In security, detection and response are often more effective, and more cost effective than more prevention (5).

Awareness and training: In security, "awareness and training" is critically important. After all, security is people-related: it is said that 70% of security problems are attributed to humans (people, process, and politics & culture). Without a security conscious and educated staff, many security measures, or much security technology, could be useless. Social engineering and taking advantage of human errors/negligence, continues to be one of the most effective attacks against information networks.

Modern protection methods and architecture: Although it is said that only 30% of security problems is related to technology, that 30% could still be significant. Choices and the adoption of appropriate modern and innovative protection technology methods and architecture, based on international and industry "best practices" and standards, could improve security substantially.

Only through such an authoritative and comprehensive program, could the information security and assurance of highly sensitive health research information be systematically and successfully protected from increasing threats and risks in the modern world.

To ensure that this strategic approach prevails, governments should enforce the scheme throughout all their agencies, and international health research communities should conclude a formal agreement to adopt standard methods and approaches. There already exist in the world a vast amount of scientific health research information not properly protected and in danger, and we must take action promptly to protect it from misuse, modification, loss/destruction, or unwarranted disclosure.

e-Health is becoming prevalent around the world, from highly sophisticated hospital information systems to Internet health portals, to telemedicine helping the poorest of countries or regions. Information security and assurance issues should seriously be addressed in e-Health (6). There are emerging communication applications on the Internet such as networked virtual offices for scientists to collaborate globally, ubiquitous RFID-based sensors to collect health data over wide areas, internationally federated identity management systems between collaborating research centers, and unimaginably powerful search engines which provide keys to almost any information people, or terrorists, are looking for. These new applications and the tremendous depository of digital information being accumulated and processed will force us to take coordinated efforts to push for a strategic approach to protecting health research information on a global scale.

Conclusion

This paper proposes a formal and comprehensive approach to protection of the security and assurance of health research information. Health research information has a high level of security requirements for: 1) confidentiality, 2) business continuity, 3) integrity, 4) quality, 5) availability, 6) authenticity, 7) accountability, 8) confidence, 9) credibility, and 10) absence of legal liability.

We believe that the approach described herein addresses collectively these issues and requirements, and facilitates a step forward toward a proactive global security process for the health research community.

Special note from authors: This paper solely reflects the views of the authors. It does not necessarily reflect the "official" views of the organization, WHO, or institutions they belong to.

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Journal of Medical Virology 75:130–136 (2005)

Protection Against Influenza Virus Infection by Intranasal Administration of Hemagglutinin Vaccine With Chitin Microparticles as an Adjuvant

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Chitin in the form of microparticles (chitin microparticles, CMP) has been demonstrated to be a potent stimulator of macrophages, promoting T-helper-1 (Th1) activation and cytokine response. In order to examine the mucosal adjuvant effect of CMP co-administered with influenza hemagglutinin (HA) vaccine against influenza infection, CMP were intranasally co-administered with influenza HA vaccine prepared from PR8 (H1N1) virus. Inoculation of the vaccine with CMP induced primary and secondary anti-HA IgA responses in the nasal wash and anti-HA IgG responses in the serum, which were significantly higher than those of nasal vaccination without CMP, and provided a complete protection against a homologous influenza virus challenge in the nasal infection influenza model. In addition, CMP-based immunization using A/Yamagata (H1N1) and A/Guizhou (H3N2) induced PR8 HA-reactive IgA in the nasal washes and specific-IgG in the serum. The immunization with A/Yamagata and CMP resulted in complete protection against a PR8 (H1N1) challenge in A/Yamagata (H1N1)-vaccinated mice, while that with A/Guizhou (H3N2) and CMP exhibited a 100-fold reduction of nasal virus titer, demonstrating the cross-protective effect of CMP and influenza vaccine. It is suggested that CMP provide a safe and effective adjuvant for nasal vaccination with inactivated influenza vaccine. ***J. Med. Virol. 75:130–136, 2005.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: influenza; chitin microparticles; nasal vaccine; adjuvant; IgA

INTRODUCTION

Effectiveness and safety are important issues to be considered in the development of a vaccine. The mucosal immune system is usually the first immunological barrier against influenza virus infections [Mestecky and McGhee, 1987]. The respiratory tract mucosa is the primary site of infection and the immunological compartment where the host immune system attacks the influenza virus. Secretory IgA antibodies are major effectors providing a front-line defense against influenza viruses in the respiratory tract mucosa [Shvartsman and Zykov, 1976; Underdown and Schiff, 1986; Murphy, 1994]. The influenza virus causes annual epidemics of influenza, largely due to the selection of new variants with mutations in the surface hemagglutinin (HA). The surface HA determines the antigenic properties of the virus and combines with sialic acid residues on epithelial cells during cell attachment [Renegar and Small, 1994]. Inactivated vaccines against the influenza virus are administered parenterally to induce serum anti-HA IgG antibodies that are highly protective against homologous virus infection, but are less effective against heterologous virus infection [Renegar and Small, 1994; Murphy and Webster, 1996]. In contrast, a large number of studies have shown that the mucosal immunity

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acquired by natural infection, which is largely due to the secreted form of IgA (s-IgA) in the respiratory tract, is more effective and provides greater cross-protection against different virus strains than the systemic immunity induced by parenteral vaccines in human [Clements et al., 1983; Couch and Kasel, 1983; Johnson et al., 1986; Murphy and Clements, 1989; Renegar and Small, 1994] and mice [Liew et al., 1984; Underdown and Schiff, 1986]. In this regard, induction of s-IgA in the RT has a great advantage in protecting against unpredictable epidemics of influenza.

It has been demonstrated that intranasal immunization with an inactivated vaccine together with cholera toxin B (CTB) subunits containing a trace amount of holotoxin (cholera toxin B*, CTB*) induces not only s-IgA with strong cross-protection against infection by virus variants of the same subtype in the upper respiratory tract, but also serum IgG with weak cross-protection against variant virus infection in the lower respiratory tract of mice [Tamura et al., 1988, 1992a,b, 1994b]. These findings were consistent with previous reports [Ramphal et al., 1979; Kris et al., 1985; Nedrud et al., 1987]. Although CTB* is an effective adjuvant for enhancing production of s-IgA, it has some adverse side effects such as producing excessive nasal discharge in humans. Adjuvants which are as effective as CTB* and safe for human use are in great demand for clinical application in nasal vaccination.

Chitin (a natural polysaccharide of *N*-acetyl-D-glucosamine) consisting of microparticles (1–20 μm in diameter) is one of the candidates for an immune enhancing adjuvant, because it can be derived from safe non-microbial sources such as shrimp, crab, and lobster. Chitin is non-allergenic, biodegradable, and biocompatible. Chitin-derived products are now used widely in the medical, veterinary, cosmetic, health supplement, and environmental industries [Okamoto et al., 1993; Strong et al., 2002]. Chitin is also a major component of fungal spores and induces a T-helper-1 (Th1) response. The innate immune system of the lung is well adapted for the clearance of airborne spores largely through phagocytosis by macrophages. This process involves secretion of IL-12 and IL-18 from the macrophages, which enhances Th1 immune responses [Strong et al., 2002]. It has been reported that the intranasal application of chitin microparticles (CMP) results in elevation of Th1 cytokines, including IL-12, IFN-γ, and TNF-α [Schaffner et al., 1982; Strong et al., 2002], and stimulation of a nasal-associated lymphoid tissue by CMP provides a bridge between the innate and adaptive immune systems [Strong et al., 2002]. Chitosan which is the partially deacetylated form of chitin has been used as a vaccine adjuvant due to its muco-adhesive properties, and has been shown to enhance antibody responses to mucosally delivered vaccine antigens [Bacon et al., 2000].

In this study, the mucosal adjuvant activity of CMP was studied when they were intranasally administered with inactivated influenza HA vaccine. It is also demonstrated that nasal CMP-based vaccine resulted in cross-protective immune responses against homologous and heterologous influenza variants.

MATERIALS AND METHODS

Hemagglutinin (HA) Vaccines and Influenza Viruses

HA vaccines (split-product virus vaccines) were prepared from the family Orthomyxoviridae, genus *Influenzavirus A,B*, species *influenzavirus A* including A/Puerto Rico/8/34 (A/PR8; H1N1), A/Yamagata/120/86 (A/Yamagata; H1N1), A/Guizhou/54/89 (A/Guizhou, H3N2) and *influenzavirus B*, B/Ibaraki/2/85 (B/Ibaraki) strains according to the method of Davenport et al. [1964] at the Kitasato Institute (Saitama, Japan). These viruses were grown in the allantoic cavities of 10–11-day fertile chicken eggs, purified and disintegrated with ethyl ether. The vaccine contains all the proteins from the virus particle; however, the major component of the vaccine is HA molecules (about 30% of the total protein). The virus, family Orthomyxoviridae, genus *Influenzavirus A,B*, species *influenzavirus A*, A/Puerto Rico/8/34 (A/PR8; H1N1) used for the challenge experiment was adapted to mice by subculturing 148 times in the ferret, 596 times in the mouse, and 73 times in 10-day fertile chicken eggs.

Adjuvants

CTB subunits containing a trace amount of holotoxin (CTB*) was prepared by adding 0.1% of holotoxin to CTB (Sigma, St. Louis, MO). The CMP were prepared by sonication of dissolved purified chitin (Sigma-Aldrich, Poole, UK) in sterile, endotoxin-free phosphate-buffered saline (PBS). The sonicated chitin particles were collected by centrifugation, washed with 70% (v/v) ethanol, and washed five times with sterile PBS to remove soluble chitin. The diameters of CMP were compared to those of standardized beads, which were 1 and 20 μm in diameter (Polysciences, Inc., Warrington, PA) by flow cytometry (FACS) analysis. The diameters of 98% of the CMP were smaller than 20 μm and 33% were less than 1 μm in size. The sterility of CMP was confirmed by plating onto agar plates, demonstrating no colony formation on the plates. The concentration of endotoxin of CMP solution was examined by a Limulus Amebocyte Lysate Assay (Bio Whittaker, Wokingham, UK) and was shown to be less than 1 EU/ml.

Immunization and Infection With Influenza Virus in Mice

Female BALB/c mice (Japan SLC, Inc., Hamamatsu, Japan), aged 6–8 weeks at the time of immunization, were used in all experiments. All animal experiments were carried out in accordance with the Guides for Animal Experiments performed at NIID and approved by the Animal Care and Use Committee of the National Institute of Infectious Diseases.

Five mice for each experimental group were anesthetized by diethyl ether and immunized primarily by dropping 5 μl of PBS containing either 1 μg of HA vaccines with 10 or 100 μg of CMP, or 1 μg of CTB* into the nostrils. The second immunization was carried out at 4 and 6 weeks later from the primary immunization (three-dose immunization protocol).

According to a modified procedure of Yetter et al. [1980] and Tamura et al. [1996, 1998], each mouse was anesthetized and infected intranasally by dropping 1.2 μl of PBS containing a virus suspension with 1×10^2 PFU of mouse-adapted PR8 virus into each nostril. As 1.2 μl of the virus suspension remained in the local nasal area, the initial viral infection was limited to the nose area.

Measurement of the Virus Titer and Anti-PR8 HA Antibodies of the Samples From the Infected Mice

After complete anesthesia with chloroform, the mice were killed. Serum and nasal wash were collected from the mice for measurement of the virus titer and antibodies against PR8 HA. The levels of IgA and IgG antibodies against HA molecules purified from the A/PR8 viruses were determined by ELISA as described previously [Tamura et al., 1996]. Briefly, ELISA was conducted sequentially from the solid phase (EIA plate; Costar, Cambridge, MA) with a ladder of reagents consisting of the following: first, HA molecules purified from the A/PR8 virus according to the procedure of Phelan et al. [1980]; second, nasal wash or serum; third, goat anti-mouse IgA antibody (α-chain specific anti-IgA antibody; Amersham Biosciences, Piscataway, NJ), or goat anti-mouse IgG antibody (γ-chain-specific anti-IgG antibody; Amersham), or anti-mouse IgG1 and IgG2a (BD Pharmingen, San Diego, CA) conjugated with biotin; fourth, streptavidin conjugated with alkaline phosphatase (Life Technologies, Rockville, MD); and finally, *p*-nitrophenylphosphate. Absorbance was measured at 405 nm using an ELISA reader. A twofold serial dilution of either purified HA-specific IgA or HA-specific monoclonal IgG (160 ng/ml) was used as a standard as described previously [Asahi et al., 2002]. The binding kinetics of the standard HA-specific monoclonal IgG was comparable to purified HA-specific IgG from immunized mice. The IgA and IgG antibody concentrations of unknown specimens were determined from the standard regression curve constructed for each assay with the programmed SJeia Autoreader (model er-8000; Sanko Jun-yaku, Tokyo Japan).

The titers of the subclasses of IgG antibodies against HA molecules were also determined by ELISA. Antibody-positive cut-off values were set at the mean ± 2 SD for pre-immune sera. The antibody titer determined by ELISA was expressed as the highest serum dilution giving a positive reaction. HA-specific monoclonal IgG1 and normal mouse serum were used as controls. The HA-specific monoclonal antibody was recognized exclusively by anti-mouse IgG1 antibody, but not by anti-mouse IgG2a antibody (Fig. 2).

The virus titer was measured as follows: each 200 μl of serial 10-fold dilutions of the nasal wash was inoculated into Madin–Darby canine kidney (MDCK) cells in a six-well plate grown in Dulbecco's modified minimum essential medium supplemented with 10% of fetal calf serum. After 1 hr incubation, each well was overlaid with 2 ml of agar medium according to the method described by Tobita et al. [1975]. The number of plaques in each well was counted at 2 days after inoculation. The experiments were repeated three to five times and the results combined. The data were represented as the mean ± SD.

Statistics

Comparisons between experimental groups were made by Student's *t*-test, and $P < 0.05$ was considered as significant.

RESULTS

Antibody Response to HA and Protection Against Virus Infection in Mice Immunized Intranasally With the HA Vaccine With Chitin Microparticles as an Adjuvant

The mucosal adjuvant efficacy of CMP for influenza HA vaccine was studied. The antibody responses against PR8 HA molecules were examined in mice immunized intranasally with PR8 vaccine together with different amounts (10 or 100 μg) of CMP or 1 μg of CTB* and boosted twice at 4 and 6 weeks after the initial immunization. The secondary anti-PR8 HA IgA antibody responses in the nasal washes and anti-PR8 HA IgG Ab responses in the serum in the immunized mice are shown in Figure 1. The adjuvant effect of CMP was enhanced with an increase of the amount of CMP (Fig. 1). The concentration of s-IgA collected from the nasal wash was more than 100 ng/μl with an average of 140 ng/μl when the mouse was immunized with 10 μg of CMP, and inoculation of 100 μg of CMP with vaccine induced an increase to over 300 ng/μl of the concentration of s-IgA in the nasal mucosa.

Meanwhile, high levels of serum anti-HA IgG responses were induced in mice given 10 μg of CMP with vaccine. The serum IgG responses seemed to parallel the s-IgA response in the nasal wash after immunization with 10 or 100 μg of CMP as adjuvants (Fig. 1). This suggests that intranasal administration of CMP with influenza HA vaccine could induce s-IgA in the nasal area as well as serum IgG.

The IgG subtypes after inoculation of CMP with the HA vaccine were examined (Fig. 2). The IgG2a titer was dramatically increased along with the increase in the amount of CMP (from 10 to 100 μg). This result was consistent with the observation that intranasal application of CMP enhanced Th1 cytokines such as IL-12, INF-γ, and TNF-α [Strong et al., 2002].

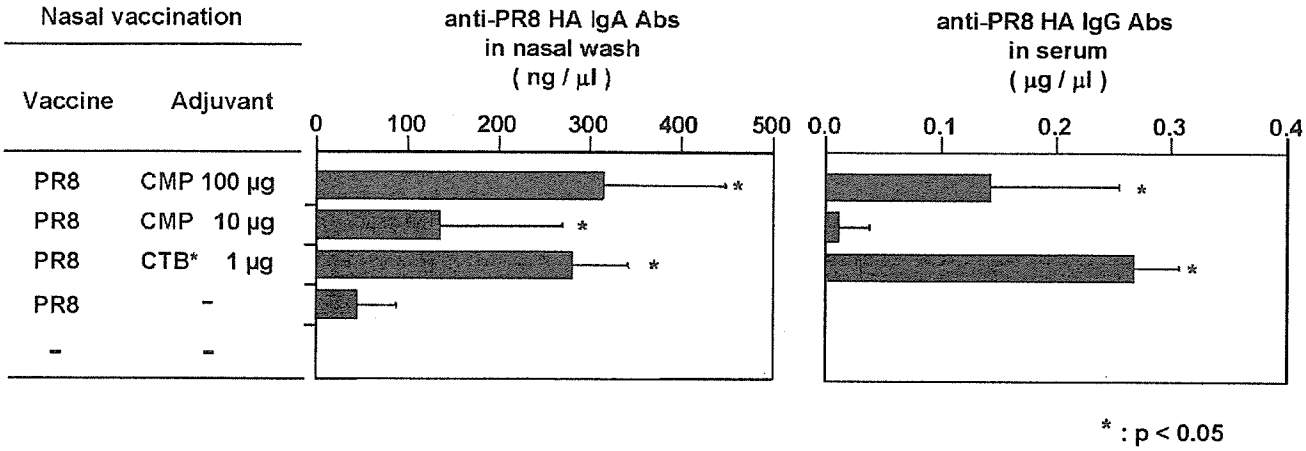

Fig. 1. Anti-PR8 hemagglutinin (HA)-specific IgA in the nasal wash and IgG in the serum from BALB/c mice immunized with intranasal vaccine with 10 or 100 μg of chitin microparticles (CMP), or 1 μg of cholera toxin B* (CTB*) as an adjuvant according to the three-dose regimen. The nasal wash and serum samples were collected at 2 weeks after the third immunization. The antibody titers of five mice from each group were measured by ELISA. The range of anti-HA IgA titers were 187.7–540.0 ng/μl (100 μg of CMP with PR8), 24.9–268.8 ng/μl (10 μg of CMP with PR8), 202.7–347.0 ng/μl (1 μg of CTB* with PR8), and 0–150.8 ng/μl (PR8 alone), respectively. The anti-HA IgA titer was not detected in the non-treated group. The range of anti-HA IgG titers were 0.0–312.1 μg/μl (100 μg of CMP with PR8), 0.0–57.86 μg/μl (10 μg of CMP with PR8), 223.5–327.2 μg/μl (1 μg of CTB* with PR8), respectively. The anti-HA IgG titer was not detected in the group treated with PR8 alone nor in the non-treated group. Each column represents mean ± SD. $^*P < 0.05$ versus the value for the group with non-immunized mice (Student's *t*-test).

Protective Efficacy Against Live Influenza Virus Challenge

The protective effect of intranasal administration of HA vaccine with CMP against influenza viral infection was studied. In control mice, virus titers were $10^{2.9}$ PFU/ml in the nasal wash at 3 days after infection with 1.2 μl of influenza virus (100 PFU) in each nostril (Fig. 3). The mice immunized with HA vaccine without CMP adjuvant showed no protective effect compared with the control mice (Fig. 3). The mice immunized with HA vaccine together with 10 or 100 μg of CMP showed complete protection against the viral challenge infection in a manner similar to the CTB*-treated group (Fig. 3). Thus, intranasal administration of HA vaccine with CMP adjuvant protected the mice against influenza virus infection. This protective effect was consistent with the enhancement of s-IgA and IgG antibody responses after inoculation of HA vaccine with CMP (Fig. 1).

Cross-Protective Effect of Influenza HA Vaccine With Chitin Microparticles as an Adjuvant

To characterize the cross-protective effects of CMP-based intranasal influenza vaccination against variant subtypes of influenza viruses, each group of mice was immunized intranasally with various vaccines (3 μg) together with CMP (100 μg) and boosted 4 and 6 weeks later. At 3 days post infection with A/PR8 (H1N1)

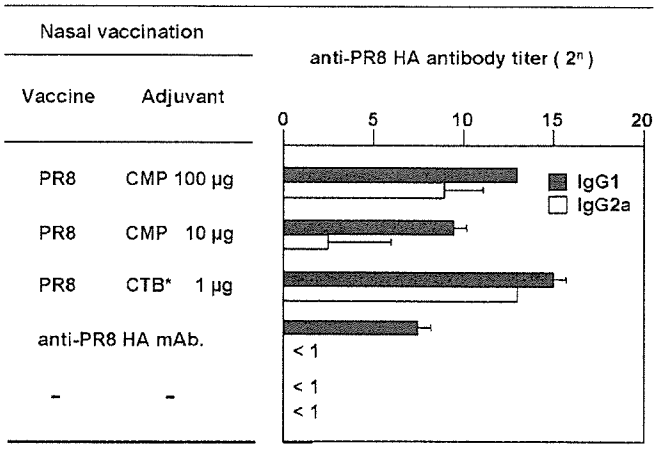

Fig. 2. Anti-PR8 HA-specific subtype of IgGs from BALB/c mice immunized with intranasal vaccine with 10 or 100 μg of CMP, or 1 μg of CTB* as an adjuvant according to the three-dose regimen. The same samples used in Figure 1 were analyzed for IgG subtypes by using IgG1- and IgG2a-specific monoclonal antibodies. HA-specific monoclonal IgG1 and normal mouse serum were used as controls. The antibody titers of five mice from each group were measured by ELISA. Each column represents mean ± SD.

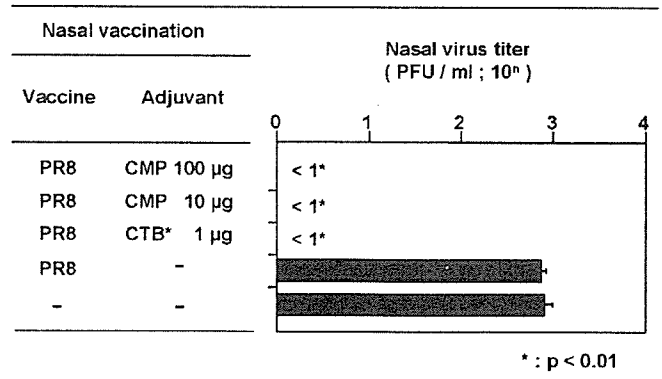

Fig. 3. Virus titers of the nasal washes from mice immunized with intranasal vaccine with 10 or 100 μg of CMP, or 1 μg of CTB* as an adjuvant according to the three-dose regimen. The mice were intranasally infected with 100 PFU of PR-8 influenza virus 2 weeks after the final immunization. The nasal washes were collected at 3 days after the virus challenge. The virus titer was measured by a plaque assay. Each column represents mean ± SD (n = 5). $^*P < 0.01$ versus the value for the group with non-immunized mice (Student's *t*-test).

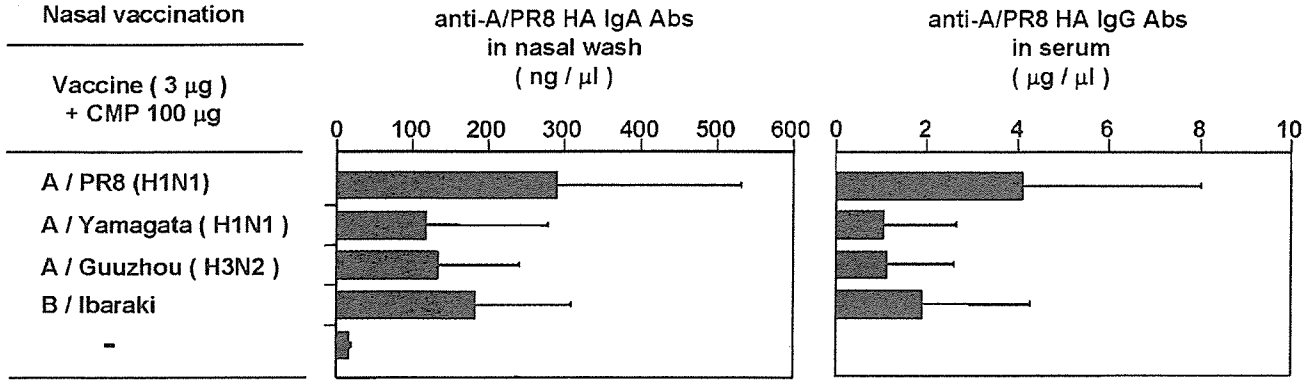

Fig. 4. Cross-protective antibody responses against PR8 HA in mice intranasally immunized with A/PR8 (H1N1), A/Yamagata (H1N1), A/Guizhou (H3N2), and B/Ibaraki vaccine with 100 μg of CMP as an adjuvant. The range of anti-A/PR8 HA IgA titers were 87.7–631.9 ng/μl (A/PR8), 19.9–356.8 ng/μl (A/Yamagata), 76.5–331.9 ng/μl (A/Guizhou), 46–346.32 ng/μl (B/Ibaraki), and 12.8–18.7 ng/μl (non-treated), respectively. The range of anti-A/PR8 HA IgG titers were 1.0–9.5 μg/μl (A/PR8), 0.0–3.4 μg/μl (A/Yamagata), 0.3–3.3 μg/μl (A/Guizhou), and 0.1–5.3 μg/μl (B/Ibaraki), respectively. The anti-A/PR8 HA IgG titer was not detected in the non-treated group. Each column represents mean ± SD (n = 5).

influenza virus performed at 2 weeks after final immunization, high s-IgA antibody responses (>200 ng/ml) in the nasal washes and high IgG antibody responses (>1 μg/ml) in the serum were observed in the mice immunized with A/PR8 virus (Fig. 4), and complete protection against the A/PR8 virus challenge was also observed (Fig. 5).

Immunization with the A/Yamagata (H1N1) nasal vaccines induced relatively low levels of nasal anti-A/PR8 HA s-IgA and serum IgG (Fig. 4), yet resulted in complete protection against challenge with 100 PFU of A/PR8 virus in 1.2 μl/nostril (Fig. 5). The mice immunized with A/Guizhou (H3N2) virus vaccine with CMP showed similarly low responses of A/PR8 HA-reactive s-IgA (<0.1 μg/ml) in the nasal wash and IgG (<1 μg/ml) in the serum as those immunized with A/Yamagata (H1N1) virus, although this group of mice still exhibited the ability to eliminate virus compared to control (100-fold reduction) after A/PR8 virus challenge (Fig. 5). Almost no protective effect was observed in mice

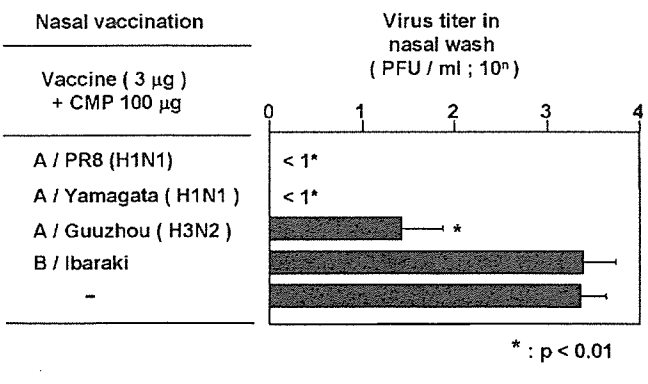

Fig. 5. Virus titers of nasal wash from mice intranasally immunized with A/PR8 (H1N1), A/Yamagata (H1N1), A/Guizhou (H3N2), and B/Ibaraki vaccine with CMP as an adjuvant. The nasal washes were collected at 3 days after the virus challenge. The virus titer was measured by a plaque assay. Each column represents mean ± SD (n = 5). *$P < 0.01$ versus the value for the group with non-immunized mice (Student's *t*-test).

intranasally immunized with B/Ibaraki vaccine, which demonstrated similar levels of A/PR8 HA-reactive s-IgA in the nasal wash and IgG in the serum as those immunized with A/Yamagata virus. These data indicate the production of cross-protective immune responses by intranasal vaccination with CMP adjuvant against homologous or heterologous virus infection in the upper respiratory tract, and the cross-reactive response was dependent on the virus strain.

DISCUSSION

Effective immunization strategies to protect against influenza virus infection involve the induction of mucosal immune responses at the nasal mucosal epithelium, which is the initial target of virus infection. To achieve effective protection against influenza infection at the mucosa, bacterial toxin-derived adjuvants such as CTB or heat-labile enterotoxin of *Escherichia coli* have been administered in conjunction with immunization [Tamura et al., 1988, 1989a,b, 1994a; Komase et al., 1998; Hagiwara et al., 1999]. Efforts to reduce the toxicity of the bacterial toxin-derived adjuvants have been carried out by using mutant toxins [Hagiwara et al., 1999, 2001] or by reducing the total amount of CTB required by adding a trace amount (0.1%) of holotoxin [Tamura et al., 1995]. Although the attenuated bacterial toxins are safe experimentally for pre-clinical animal models, it is still somewhat problematic for administration in human vaccination. An effective and safe adjuvant for intranasal vaccination in humans will be of great value. In this study, a new adjuvant system for intranasal vaccination without bacterial toxins or their derivatives is described.

CMP as an adjuvant is comparable to CTB* in enhancing anti-HA antibodies when administered intranasally with vaccine. Nasal immunization of vaccine and CMP exhibited not only an increase in mucosal s-IgA, but also a high titer of anti-HA IgG in the serum, and provided complete protection against viral infection at a level

comparable to CTB* (Fig. 3). Thus, CMP seem to be an effective adjuvant for a nasal influenza vaccine.

The advantage of the nasal route of vaccination for influenza is the induction of s-IgA at the mucosal epithelium, which elicits cross-protective immunity more effectively than serum IgG [Tamura et al., 1992a]. In fact, we have observed the cross-protective effect of CMP combined with vaccine against various strains of influenza virus, including A/PR8 (H1N1), A/Yamagata (H1N1), A/Guizhou (H3N2), and B/Ibaraki. The PR8 HA-reactive secretary IgA in the nasal wash and serum IgG were detected in the mice immunized with the same H1N1 virus strain, A/Yamagata, and a complete protective effect against viral challenge was observed in this group. In addition, immunization of the H3N2 strain, A/Guizhou, produced a similar level of secretory IgA and serum IgG as those of A/Yamagata, and exhibited a modest viral protection effect which was a 100-fold reduction of viral titer compared to that of the non-treated group. Thus, production of cross-reactive s-IgA caused homologous and heterologous protection against viral infection, although other mechanisms might be involved in addition to cross-reactive protection.

Although the detailed mechanism of the adjuvant effect of CMP is still unclear, intranasal application of small doses (10–100 μg) of CMP has been shown to result in elevation of Th1 cytokines, such as IL-12, IFN-γ, and TNF-α, and reduction in IL-4 production during allergen challenge [Strong et al., 2002]. It has been reported that immunization with chitin increased Th1 responses in spleen cells, delayed-type hypersensitivity reactions, and serum IgG2a levels along with decreases of Th2 responses [Shibata et al., 2001]. Furthermore, oral administration of chitin has been reported to decrease IgE levels and lung eosinophil numbers, and inhibit Th2 cytokine response [Shibata et al., 2000]. The Th1 immunostimulatory properties induced by vaccine and CMP were affirmed by the enhancement of IgG2a response in a CMP dose-dependent manner (Fig. 2).

It is necessary for the development of a prophylactic vaccine that both vaccine and adjuvant are safe for use in humans. CMP, which are already used widely as medical supplements [Okamoto et al., 1993; Strong et al., 2002], might provide an alternative to microbial-derived adjuvants such as CTB. An intranasal vaccination protocol consisting of an influenza HA vaccine with CMP is described as an effective and safe adjuvant in a three-dose vaccination strategy to vaccinate against influenza. Further studies are needed to determine if such a nasal vaccine would be effective in humans.

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Clinicopathological and virological analyses of familial human T-lymphotropic virus type I–associated polyneuropathy

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Human T-lymphotropic virus type I (HTLV-I) is known to be the causative agent of the chronic myelopathy, HTLV-I–associated myelopathy (HAM), and on rare occasions infection is also associated with the development of polyneuropathy. Here the authors present an HTLV-I–positive family of whom four members developed a chronic demyelinating polyneuropathy without HAM. Four female patients in a family from Hokkaido in Japan developed distal dominant paresthesia and muscle weakness in the second and third decades of their life. Neurological findings at ages ranging from 50 to 65 years included mild painful sensorimotor disturbances with atrophy of the distal parts of the extremities but without pyramidal signs or hyperactive tendon reflexes. Magnetic resonance imaging (MRI) findings of brain and spinal cord were unremarkable. Serum HTLV-I antibody levels were elevated at 1:8,192 to 1:32,768, whereas those in cerebrospinal fluid were low at 1:4 to 1:8. Electrophysiological studies revealed polyphasic compound muscle action potentials with denervation potentials on nerve conduction studies and neurogenic patterns by electromyography, which were consistent with signs of chronic motor dominant demyelinating polyneuropathy. Sural nerve biopsy showed decreased myelinated fibers, occurrence of globule formation, myelin ovoid and remyelinated fibers, and an infiltration of CD68-positive macrophages with occasional CD4-positive T cells in the nerve fascicles. The polyneuropathy was responsive to steroid therapy. Analyses of serological human leukocyte antigen (HLA) types indicated that none of the patients possessed a high-risk HLA type known to be associated with adult T-cell leukemia (ATL), whereas they did have high responsive alleles to HTLV-I *env* similar to that observed in HAM. Nucleotide sequence analysis of the HTLV-I tax region demonstrated the B subgroup in all patients. This study suggests that HTLV-I infection can result in the development of a familial form of polyneuropathy that is associated with distinct HLA class I alleles, which might possibly involve a distinct virus subtype. *Journal of NeuroVirology* (2005) 11, 199–207.

Keywords: familial polyneuropathy; HTLV-1; immunostaining

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Introduction

Human T-lymphotropic virus type I (HTLV-I) is known to be associated with a range of lymphoproliferative and inflammatory disorders. The former, adult T-cell leukemia/lymphoma (ATLL) is a spectrum of malignancies of mature CD4-positive T lymphocytes (Johnson *et al*, 2001). The major inflammatory disorder is neurological, and is a chronic encephalomyelopathy known both as tropical spastic paraparesis (TSP) and HTLV-I–associated myelopathy (HAM) (Gessain *et al*, 1985; Osame *et al*, 1986). These two disorders are the most representative of HTLV-I–associated processes affecting the blood and the central nervous system (CNS), respectively (Nagai and Jacobson, 2001; Yoshida, 2001). In recent years, additional inflammatory disorders that have been associated with HTLV-I infection have included uveitis, arthropathy, alveolitis, dermatitis, Sjögren's syndrome (Yamaguchi and Takatsuki, 1993), and myositis (Douen *et al*, 1997; Vernant *et al*, 1990).

Involvement of the peripheral nerves has been reported in association with both the smoldering and acute forms of ATLL (Cabrera *et al*, 1991; Hori *et al*, 1998; Kasahata *et al*, 2000; Kuroda *et al*, 1989; Mitsui *et al*, 1999; Murata *et al*, 1990; Nakano *et al*, 1991; Okamura *et al*, 1984), and polyneuropathy has also been described in a number of patients with TSP/HAM (Arimura *et al*, 1987; Bhigjee *et al*, 1993; Funamoto *et al*, 1989; Johkura *et al*, 1995; Kohriyama *et al*, 1992; Kyuno *et al*, 1993; Nakazato *et al*, 1989; Said *et al*, 1988; Sugimura *et al*, 1990; Vallat *et al*, 1993; Vernant *et al*, 1990; Yanagihara *et al*, 1999). However, there have only been a few reports of chronic polyneuropathy occurring in HTLV-I–asymptomatic carriers (Arakawa *et al*, 1990; Douen *et al*, 1997; Hori *et al*, 1998; Kanzaki *et al*, 1995). In this study, we have identified four HTLV-I–seropositive patients in a family, all of whom developed a chronic demyelinating polyneuropathy without HAM/TSP. The results of the clinical evaluation, together with immunological, histopathological, and molecular genetic studies, suggest the existence of a rare form of a familial polyneuropathy associated with HTLV-I infection.

Results

The clinical features of all four patients are summarized in Table 1. The exact age of onset of symptoms could not be accurately determined as the initial neurological symptoms were often vague, involving numbness or painful weakness of the lower extremities. However, in two patients (cases 1 and 3) initial complaints seemingly began at the end of their 2nd decade of life, and these had progressed slowly without significant disabilities in that the patients were able to work normally. The other two patients (cases 2 and 4) developed neurological abnormalities at 46 and 45 years old, 7 and 5 years, respectively, prior to their initial neurological examination.

Serological studies demonstrated high levels of antibody against HTLV-I in all four patients, with titers ranging from 1:8,192 to 1:32,768 (positive: greater than 1:16), whereas cerebrospinal fluid (CSF) were low from 1:4 to 1:8. Other viral antibody titers including human immunodeficiency virus (HIV) were all negative both in sera and CSFs. There were no high protein levels in the CSF.

Electrophysiological studies with proximal stimulation demonstrated that the compound muscle action potentials (CMAPs) exhibited a temporal dispersion in all the nerves but primarily in the tibial nerve, as exemplified in case 3 (Figure 1a). Sensory as well as motor nerve conduction studies (NCSs) and F-wave were grossly within normal ranges and as was the somatosensory evoked potential (SSEP).

Table 1 Clinical summary of four cases in a family

Family A			*HTLV-I titers (PA)*						
Case	*Age*	*Sex*	*Neurologic signs*	*Onset (y)*	*Serum*	*CSF*	*NCS/EMG*	*HLA*	*Note*
1	61 y	F	Paresthesia, distal motor weakness	2nd decade	16,384×	4×	Motor dominant CDP, neurogenic pattern	A33, 68 B44, 51 DR9, 12	Uveitis, thyroid Ca, deafness
2	53 y	F	Paresthesia, distal	4th decade	8,192×	8×	Motor dominant CDP, neurogenic pattern	A2, 68 B51 DR4, 9	43 y, uterus Ca
3	52 y	F	Paresthesia, distal	2nd decade	32,768×	8×	Motor dominant CDP, neurogenic pattern	A2, 33 B39, 48, Cw7 DR2, 11	Arthralgia, HBV(+)
4	50 y	F	Paresthesia, distal motor weakness	3rd decade	8,192×	8×	Motor dominant CDP, neurogenic pattern	A24, 33 B54, 39, Cw1, 7 DR2, 4	12 y pulmonary tuberculosis

CSF: cerebrospinal fluid; NCS: nerve conduction studies; EMG: electromyography; CDP: chronic demyelinating polyneuropathy; PA: particle agglutination method; HBV: hepatitis B virus; Ca: carcinoma.

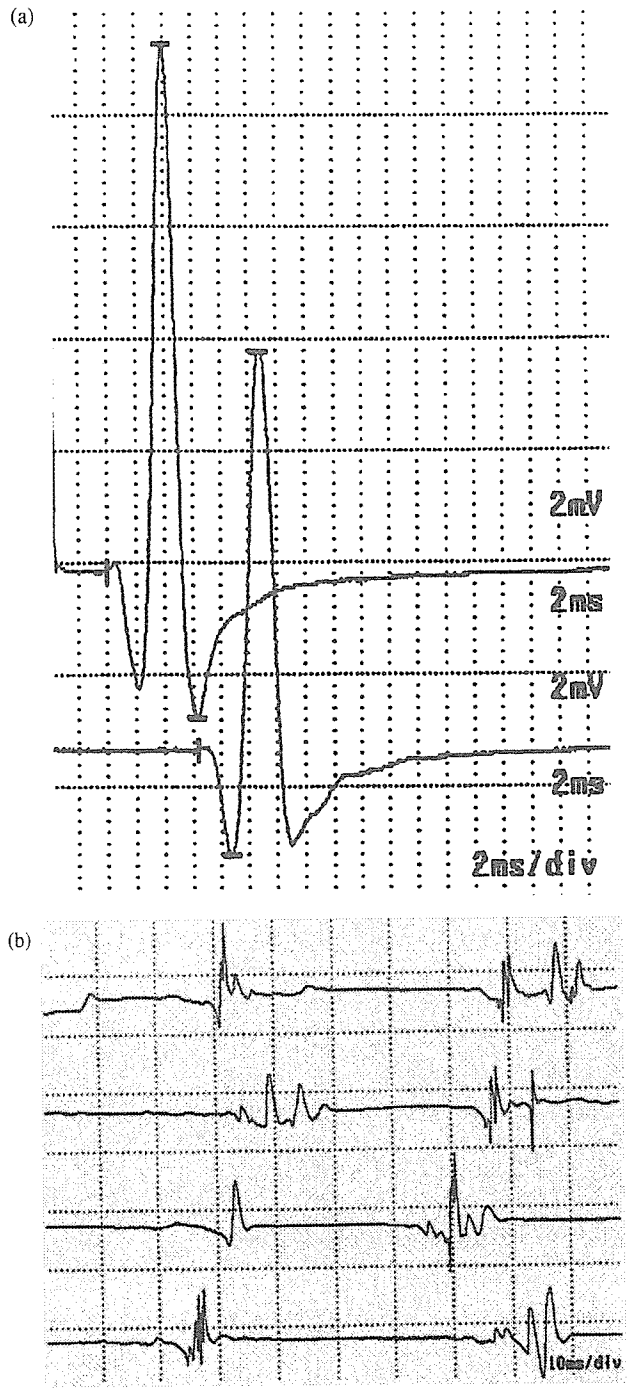

Figure 1 Electrophysiological findings of case 3 (**a**) and case 1 (**b**). **a**, Compound muscle action potentials (CAMPs) of right tibial nerve of, showing temporal dispersion. **b**, EMG of the right anterior tibial muscle, showing polyphasic potentials with occasional long duration potentials.

On electromusculographic (EMG) recordings at mild contraction, polyphasic potentials with decreased interference were detected predominantly in the lower extremities, which indicated a chronic motor dominant demyelinating polyneuropathy (case 1) (Figure 1b). There were no myopathic changes on EMG.

Histopathological findings of the sural nerves revealed decreased myelinated fibers with round bodies containing degenerated material compatible with so-called myelin ovoid, and thinly myelinated axons, in case 1 by toluidine blue (TB) staining (Figure 2a and b). Myelin globules were also occasionally encountered (Figure 2c). In cases 2 and 3, a slight decrease of nerve fibers was evident with a few thinly myelinated axons, but globular bodies were not observed. Aggregation of thinly myelinated axons was observed in all three cases examined (Figure 2d), suggestive of remyelination. No axonal changes were observed. Lymphocytic infiltration was not observed in TB-staining preparations. The sural nerve of case 4 was not examined by TB staining. By immunostaining, there was rare infiltration of CD45RO-positive T cells into the nerve fascicles and/or vessels around the nerve in all four cases. These T cells were mostly characterised as the CD4 positive (Figure 3a). CD68-positive macrophages were almost exclusively found among the nerve fibers (Figure 3b). There were no inflammatory features such as perivascular lymphocytic infiltration or neoplastic T cells in the skin and muscles in any of the biopsy samples.

The serological human leukocyte antigen (HLA) subtype is summarized in Table 1. All four patients did not have such an HLA type as A26 that have been reported to be associated with ATLL. Mutations in the peripheral myelin protein-22 (PMP22) gene and myelin protein zero (P0) gene were not detected in any of the patients.

Nucleotide sequence analysis of the HTLV-I tax region following amplification using polymerase chain reaction (PCR) demonstrated that the nucleotide position (np) and amino acid change were C in np7898 and np7959, G in np8208, and A in np8344, confirming that all four patients were infected with the tax B subgroup, which belongs to the cosmopolitan B subgroup of HTLV-I (Furukawa *et al*, 2000).

Discussion

HTLV-I–related neuropathies have been reported by a number of authors following the initial description of TSP/HAM. A summary of cases described in the literature is shown in Table 2. It can be seen that in most cases a diagnosis of HAM had been initially made before signs of neuropathy were evident. Arimura *et al* (1987), using electrophysiologic studies, first described the involvement of peripheral nerves in HAM patients. In a familial case of HAM, Nakazato *et al* (1989) described sural nerve pathology, and proposed that HLTV-I infection need not be limited to disorders involving the spinal cord but could also involve peripheral nerves. Polyneuropathy in the absence of HAM has been rarely reported. In the first report Arakawa *et al* (1990) described