SB202190, which showed no inhibitory effect on PV and EV71 infections (data not shown). Infact, the *in vivo* effect of GW5074 is enigmatic: GW5074 has a neuroprotective action in neurones by activating, but not by inhibiting, B-Raf (Chen *et al.*, 2008). This action was independent of the MEK/ERK signalling pathway, but depended on down-regulation of ATF-3 mRNA (Chen *et al.*, 2008). However, siRNA treatments against these putative targets did not consistently suppress PV replication (Fig. 6a). Interestingly, one set of siRNA against MST2 (siRNA03) showed a partial inhibitory effect without inducing an apparent interferon response. However, other sets of siRNA against MST2 failed to show the inhibitory effect, and one set (siRNA01) rather stimulated the infection (Fig. 6a). Therefore, the target of GW5074 for its inhibitory effect seemed different from those of *in vivo* neuroprotective action (B-Raf and ATF-3), but should be conserved among the enterovirus infections. It is also possible that GW5074 reacted with highly conserved region of the viral proteins for its inhibitory effect. The identity of the target of GW5074 in the inhibitory effect of PV replication remained to be further identified.

In summary, we have characterized four compounds with high anti-EV71 and/or anti-PV activity. Live and inactivated vaccines have been established for PV and used for global eradication of poliomyelitis (Sabin, 1965; Salk *et al.*, 1954). However, there is no effective therapy for vaccine-associated paralytic poliomyelitis, which is caused by virulent revertants of vaccine strains at a rate of one case per 520 000 doses associated with the first dose of the vaccine (Nkowane *et al.*, 1987). For EV71, various vaccine candidates and therapies are being developed, but no vaccine has been established (Chen *et al.*, 2006; Chiu *et al.*, 2006; Liu *et al.*, 2005, 2007; Shih *et al.*, 2004; Tan & Cardosa, 2007; Tung *et al.*, 2007; Wu *et al.*, 2007; Yu *et al.*, 2000). The results obtained in this study would be useful for the identification of novel targets to develop anti-enterovirus drugs that could serve as therapeutic and/or prophylactic agents against acute neurological diseases caused by enterovirus infection.

ACKNOWLEDGEMENTS

We are grateful to Junko Wada for her excellent technical assistance. We are grateful to Atsushi Kato for kindly providing luciferase-encoding SeV. This study was supported in part by Grants-in-Aid for the Promotion of Polio Eradication and Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labour and Welfare, and by Grant-in-Aid for Young Scientists from the Ministry of Education, Cluture, Science and Technology (KAKENHI 19790347).

REFERENCES

Abdel-Rahman, S. M. & Kearns, G. L. (1998). Single-dose pharmacokinetics of a pleconaril (VP63843) oral solution and effect of food. *Antimicrob Agents Chemother* **42**, 2706–2709.

Arita, M., Nagata, N., Sata, T., Miyamura, T. & Shimizu, H. (2006). Quantitative analysis of poliomyelitis-like paralysis in mice induced by a poliovirus replicon. *J Gen Virol* **87**, 3317–3327.

Arita, M., Nagata, N., Iwata, N., Ami, Y., Suzaki, Y., Mizuta, K., Iwasaki, T., Sata, T., Wakita, T. & Shimizu, H. (2007). An attenuated strain of enterovirus 71 belonging to genotype A showed a broad spectrum of antigenicity with attenuated neurovirulence in cynomolgus monkeys. *J Virol* **81**, 9386–9395.

Arita, M., Ami, Y., Wakita, T. & Shimizu, H. (2008). Cooperative effect of the attenuation determinants derived from poliovirus Sabin 1 strain is essential for attenuation of enterovirus 71 in the NOD/SCID mouse infection model. *J Virol* **82**, 1787–1797.

Bain, J., Plater, L., Elliott, M., Shpiro, N., Hastie, J., McLauchlan, H., Klevernic, I., Arthur, S., Alessi, D. & Cohen, P. (2007). The selectivity of protein kinase inhibitors: a further update. *Biochem J* **408**, 297–315.

Bedard, K. M., Daijogo, S. & Semler, B. L. (2007). A nucleo-cytoplasmic SR protein functions in viral IRES-mediated translation initiation. *EMBO J* **26**, 459–467.

Belov, G. A., Habbersett, C., Franco, D. & Ehrenfeld, E. (2007). Activation of cellular Arf GTPases by poliovirus protein 3CD correlates with virus replication. *J Virol* **81**, 9259–9267.

Blondel, B., Crainic, R., Fichot, O., Dufraisse, G., Candrea, A., Diamond, D., Girard, M. & Horaud, F. (1986). Mutations conferring resistance to neutralization with monoclonal antibodies in type 1 poliovirus can be located outside or inside the antibody-binding site. *J Virol* **57**, 81–90.

Bodian, D. (1949). Histopathologic basis of clinical findings in poliomyelitis. *Am J Med* **6**, 563–578.

Bubeck, D., Filman, D. J. & Hogle, J. M. (2005). Cryo-electron microscopy reconstruction of a poliovirus-receptor-membrane complex. *Nat Struct Mol Biol* **12**, 615–618.

Chang, L. Y., Lin, T. Y., Hsu, K. H., Huang, Y. C., Lin, K. L., Hsueh, C., Shih, S. R., Ning, H. C., Hwang, M. S. & other authors (1999). Clinical features and risk factors of pulmonary oedema after enterovirus-71-related hand, foot, and mouth disease. *Lancet* **354**, 1682–1686.

Chen, H. F., Chang, M. H., Chiang, B. L. & Jeng, S. T. (2006). Oral immunization of mice using transgenic tomato fruit expressing protein VP1 from enterovirus 71. *Vaccine* **24**, 2944–2951.

Chen, H. M., Wang, L. & D'Mello, S. R. (2008). Inhibition of ATF-3 expression by B-Raf mediates the neuroprotective action of GW5074. *J Neurochem* **105**, 1300–1312.

Chiu, C. H., Chu, C., He, C. C. & Lin, T. Y. (2006). Protection of neonatal mice from lethal enterovirus 71 infection by maternal immunization with attenuated *Salmonella enterica* serovar Typhimurium expressing VP1 of enterovirus 71. *Microbes Infect* **8**, 1671–1678.

Chumakov, M., Voroshilova, M., Shindarov, L., Lavrova, I., Gracheva, L., Koroleva, G., Vasilenko, S., Brodvarova, I., Nikolova, M. & other authors (1979). Enterovirus 71 isolated from cases of epidemic poliomyelitis-like disease in Bulgaria. *Arch Virol* **60**, 329–340.

Crotty, S., Cameron, C. E. & Andino, R. (2001). RNA virus error catastrophe: direct molecular test by using ribavirin. *Proc Natl Acad Sci U S A* **98**, 6895–6900.

Crotty, S., Hix, L., Sigal, L. J. & Andino, R. (2002). Poliovirus pathogenesis in a new poliovirus receptor transgenic mouse model: age-dependent paralysis and a mucosal route of infection. *J Gen Virol* **83**, 1707–1720.

Crotty, S., Saleh, M. C., Gitlin, L., Beske, O. & Andino, R. (2004). The poliovirus replication machinery can escape inhibition by an antiviral drug that targets a host cell protein. *J Virol* **78**, 3378–3386.

de la Torre, J. C., Giachetti, C., Semler, B. L. & Holland, J. J. (1992). High frequency of single-base transitions and extreme frequency of

precise multiple-base reversion mutations in poliovirus. *Proc Natl Acad Sci U S A* 89, 2531–2535.

Diamond, D. C., Jameson, B. A., Bonin, J., Kohara, M., Abe, S., Itoh, H., Komatsu, T., Arita, M., Kuge, S. & other authors (1985). Antigenic variation and resistance to neutralization in poliovirus type 1. *Science* 229, 1090–1093.

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. & Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411, 494–498.

Esfandiarei, M., Luo, H., Yanagawa, B., Suarez, A., Dabiri, D., Zhang, J. & McManus, B. M. (2004). Protein kinase B/Akt regulates coxsackievirus B3 replication through a mechanism which is not caspase dependent. *J Virol* 78, 4289–4298.

Geller, R., Vignuzzi, M., Andino, R. & Frydman, J. (2007). Evolutionary constraints on chaperone-mediated folding provide an antiviral approach refractory to development of drug resistance. *Genes Dev* 21, 195–205.

Gradi, A., Svitkin, Y. V., Imataka, H. & Sonenberg, N. (1998). Proteolysis of human eukaryotic translation initiation factor eIF4GII, but not eIF4GI, coincides with the shutoff of host protein synthesis after poliovirus infection. *Proc Natl Acad Sci U S A* 95, 11089–11094.

Graham, F. L., Smiley, J., Russell, W. C. & Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol* 36, 59–74.

Hasan, M. K., Kato, A., Shioda, T., Sakai, Y., Yu, D. & Nagai, Y. (1997). Creation of an infectious recombinant Sendai virus expressing the firefly luciferase gene from the 3′ proximal first locus. *J Gen Virol* 78, 2813–2820.

Hellen, C. U., Witherell, G. W., Schmid, M., Shin, S. H., Pestova, T. V., Gil, A. & Wimmer, E. (1993). A cytoplasmic 57-kDa protein that is required for translation of picornavirus RNA by internal ribosomal entry is identical to the nuclear pyrimidine tract-binding protein. *Proc Natl Acad Sci U S A* 90, 7642–7646.

Ho, M., Chen, E. R., Hsu, K. H., Twu, S. J., Chen, K. T., Tsai, S. F., Wang, J. R. & Shih, S. R. (1999). An epidemic of enterovirus 71 infection in Taiwan. *N Engl J Med* 341, 929–935.

Huang, C. C., Liu, C. C., Chang, Y. C., Chen, C. Y., Wang, S. T. & Yeh, T. F. (1999). Neurologic complications in children with enterovirus 71 infection. *N Engl J Med* 341, 936–942.

Hunt, S. L., Hsuan, J. J., Totty, N. & Jackson, R. J. (1999). unr, a cellular cytoplasmic RNA-binding protein with five cold-shock domains, is required for internal initiation of translation of human rhinovirus RNA. *Genes Dev* 13, 437–448.

Ida-Hosonuma, M., Iwasaki, T., Taya, C., Sato, Y., Li, J., Nagata, N., Yonekawa, H. & Koike, S. (2002). Comparison of neuropathogenicity of poliovirus in two transgenic mouse strains expressing human poliovirus receptor with different distribution patterns. *J Gen Virol* 83, 1095–1105.

Irurzun, A., Perez, L. & Carrasco, L. (1992). Involvement of membrane traffic in the replication of poliovirus genomes: effects of brefeldin A. *Virology* 191, 166–175.

Kärber, G. (1931). Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. *Naunyn Schmiedebergs Arch Pharmacol* 162, 480–483.

Koike, S., Taya, C., Aoki, J., Matsuda, Y., Ise, I., Takeda, H., Matsuzaki, T., Amanuma, H., Yonekawa, H. & Nomoto, A. (1994). Characterization of three different transgenic mouse lines that carry human poliovirus receptor gene – influence of the transgene expression on pathogenesis. *Arch Virol* 139, 351–363.

Komatsu, H., Shimizu, Y., Takeuchi, Y., Ishiko, H. & Takada, H. (1999). Outbreak of severe neurologic involvement associated with enterovirus 71 infection. *Pediatr Neurol* 20, 17–23.

Krausslich, H. G., Nicklin, M. J., Toyoda, H., Etchison, D. & Wimmer, E. (1987). Poliovirus proteinase 2A induces cleavage of eucaryotic initiation factor 4F polypeptide p220. *J Virol* 61, 2711–2718.

Lackey, K., Cory, M., Davis, R., Frye, S. V., Harris, P. A., Hunter, R. N., Jung, D. K., McDonald, O. B., McNutt, R. W. & other authors (2000). The discovery of potent cRaf1 kinase inhibitors. *Bioorg Med Chem Lett* 10, 223–226.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.

Liu, M. L., Lee, Y. P., Wang, Y. F., Lei, H. Y., Liu, C. C., Wang, S. M., Su, I. J., Wang, J. R., Yeh, T. M. & other authors (2005). Type I interferons protect mice against enterovirus 71 infection. *J Gen Virol* 86, 3263–3269.

Liu, C. C., Lian, W. C., Butler, M. & Wu, S. C. (2007). High immunogenic enterovirus 71 strain and its production using serum-free microcarrier Vero cell culture. *Vaccine* 25, 19–24.

Lum, L. C., Wong, K. T., Lam, S. K., Chua, K. B., Goh, A. Y., Lim, W. L., Ong, B. B., Paul, G., AbuBakar, S. & Lambert, M. (1998). Fatal enterovirus 71 encephalomyelitis. *J Pediatr* 133, 795–798.

Luo, H., Yanagawa, B., Zhang, J., Luo, Z., Zhang, M., Esfandiarei, M., Carthy, C., Wilson, J. E., Yang, D. & McManus, B. M. (2002). Coxsackievirus B3 replication is reduced by inhibition of the extracellular signal-regulated kinase (ERK) signaling pathway. *J Virol* 76, 3365–3373.

Macejak, D. G. & Sarnow, P. (1992). Association of heat shock protein 70 with enterovirus capsid precursor P1 in infected human cells. *J Virol* 66, 1520–1527.

MacLennan, C., Dunn, G., Huissoon, A. P., Kumararatne, D. S., Martin, J., O'Leary, P., Thompson, R. A., Osman, H., Wood, P. & other authors (2004). Failure to clear persistent vaccine-derived neurovirulent poliovirus infection in an immunodeficient man. *Lancet* 363, 1509–1513.

Maynell, L. A., Kirkegaard, K. & Klymkowsky, M. W. (1992). Inhibition of poliovirus RNA synthesis by brefeldin A. *J Virol* 66, 1985–1994.

McMinn, P. C. (2002). An overview of the evolution of enterovirus 71 and its clinical and public health significance. *FEMS Microbiol Rev* 26, 91–107.

Meerovitch, K., Svitkin, Y. V., Lee, H. S., Lejbkowicz, F., Kenan, D. J., Chan, E. K., Agol, V. I., Keene, J. D. & Sonenberg, N. (1993). La autoantigen enhances and corrects aberrant translation of poliovirus RNA in reticulocyte lysate. *J Virol* 67, 3798–3807.

Mendelsohn, C. L., Wimmer, E. & Racaniello, V. R. (1989). Cellular receptor for poliovirus: molecular cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily. *Cell* 56, 855–865.

Minor, P. D. (1992). The molecular biology of polio vaccines. *J Gen Virol* 73, 3065–3077.

Nagata, N., Shimizu, H., Ami, Y., Tano, Y., Harashima, A., Suzaki, Y., Sato, Y., Miyamura, T., Sata, T. & Iwasaki, T. (2002). Pyramidal and extrapyramidal involvement in experimental infection of cynomolgus monkeys with enterovirus 71. *J Med Virol* 67, 207–216.

Nijhuis, M., van Maarseveen, N., Schuurman, R., Verkuijlen, S., de Vos, M., Hendriksen, K. & van Loon, A. M. (2002). Rapid and sensitive routine detection of all members of the genus enterovirus in different clinical specimens by real-time PCR. *J Clin Microbiol* 40, 3666–3670.

Nkowane, B. M., Wassilak, S. G., Orenstein, W. A., Bart, K. J., Schonberger, L. B., Hinman, A. R. & Kew, O. M. (1987). Vaccine-associated paralytic poliomyelitis. United States: 1973 through 1984. *JAMA* 257, 1335–1340.

Pfister, T. & Wimmer, E. (1999). Characterization of the nucleoside triphosphatase activity of poliovirus protein 2C reveals a mechanism

by which guanidine inhibits poliovirus replication. *J Biol Chem* **274**, 6992–7001.

Pincus, S. E., Diamond, D. C., Emini, E. A. & Wimmer, E. (1986). Guanidine-selected mutants of poliovirus: mapping of point mutations to polypeptide 2C. *J Virol* **57**, 638–646.

Pincus, S. E., Rohl, H. & Wimmer, E. (1987). Guanidine-dependent mutants of poliovirus: identification of three classes with different growth requirements. *Virology* **157**, 83–88.

Porter, D. C., Ansardi, D. C., Wang, J., McPherson, S., Moldoveanu, Z. & Morrow, C. D. (1998). Demonstration of the specificity of poliovirus encapsidation using a novel replicon which encodes enzymatically active firefly luciferase. *Virology* **243**, 1–11.

Ranganathan, S., Singh, S., Poh, C. L. & Chow, V. T. (2002). The hand, foot and mouth disease virus capsid: sequence analysis and prediction of antigenic sites from homology modelling. *Appl Bioinformatics* **1**, 43–52.

Ren, R. & Racaniello, V. R. (1992). Human poliovirus receptor gene expression and poliovirus tissue tropism in transgenic mice. *J Virol* **66**, 296–304.

Sabin, A. B. (1965). Oral poliovirus vaccine. History of its development and prospects for eradication of poliomyelitis. *JAMA* **194**, 872–876.

Salk, J. E., Bazeley, P. L., Bennett, B. L., Krech, U., Lewis, L. J., Ward, E. N. & Youngner, J. S. (1954). Studies in human subjects on active immunization against poliomyelitis. II. A practical means for inducing and maintaining antibody formation. *Am J Public Health Nations Health* **44**, 994–1009.

Sanford, J. R., Gray, N. K., Beckmann, K. & Caceres, J. F. (2004). A novel role for shuttling SR proteins in mRNA translation. *Genes Dev* **18**, 755–768.

Shih, S. R., Tsai, M. C., Tseng, S. N., Won, K. F., Shia, K. S., Li, W. T., Chern, J. H., Chen, G. W., Lee, C. C. & other authors (2004). Mutation in enterovirus 71 capsid protein VP1 confers resistance to the inhibitory effects of pyridyl imidazolidinone. *Antimicrob Agents Chemother* **48**, 3523–3529.

Shimizu, H., Agoh, M., Agoh, Y., Yoshida, H., Yoshii, K., Yoneyama, T., Hagiwara, A. & Miyamura, T. (2000). Mutations in the 2C region of poliovirus responsible for altered sensitivity to benzimidazole derivatives. *J Virol* **74**, 4146–4154.

Si, X., Luo, H., Morgan, A., Zhang, J., Wong, J., Yuan, J., Esfandiarei, M., Gao, G., Cheung, C. & McManus, B. M. (2005). Stress-activated protein kinases are involved in coxsackievirus B3 viral progeny release. *J Virol* **79**, 13875–13881.

Tan, C. S. & Cardosa, M. J. (2007). High-titred neutralizing antibodies to human enterovirus 71 preferentially bind to the N-terminal portion of the capsid protein VP1. *Arch Virol* **152**, 1069–1073.

Teterina, N. L., Gorbalenya, A. E., Egger, D., Bienz, K. & Ehrenfeld, E. (1997). Poliovirus protein 2C determinants of membrane binding and rearrangements in mammalian cells. *J Virol* **71**, 8962–8972.

Tung, W. S., Bakar, S. A., Sekawi, Z. & Rosli, R. (2007). DNA vaccine constructs against enterovirus 71 elicit immune response in mice. *Genet Vaccines Ther* **5**, 6.

Vance, L. M., Moscufo, N., Chow, M. & Heinz, B. A. (1997). Poliovirus 2C region functions during encapsidation of viral RNA. *J Virol* **71**, 8759–8765.

Walter, B. L., Nguyen, J. H., Ehrenfeld, E. & Semler, B. L. (1999). Differential utilization of poly(rC) binding protein 2 in translation directed by picornavirus IRES elements. *RNA* **5**, 1570–1585.

Wang, S. M., Liu, C. C., Tseng, H. W., Wang, J. R., Huang, C. C., Chen, Y. J., Yang, Y. J., Lin, S. J. & Yeh, T. F. (1999). Clinical spectrum of enterovirus 71 infection in children in southern Taiwan, with an emphasis on neurological complications. *Clin Infect Dis* **29**, 184–190.

Wang, S. M., Lei, H. Y., Huang, K. J., Wu, J. M., Wang, J. R., Yu, C. K., Su, I. J. & Liu, C. C. (2003). Pathogenesis of enterovirus 71 brainstem encephalitis in pediatric patients: roles of cytokines and cellular immune activation in patients with pulmonary edema. *J Infect Dis* **188**, 564–570.

Wessels, E., Duijsings, D., Niu, T. K., Neumann, S., Oorschot, V. M., de Lange, F., Lanke, K. H., Klumperman, J., Henke, A. & other authors (2006). A viral protein that blocks Arf1-mediated COP-I assembly by inhibiting the guanine nucleotide exchange factor GBF1. *Dev Cell* **11**, 191–201.

Wu, T. C., Wang, Y. F., Lee, Y. P., Wang, J. R., Liu, C. C., Wang, S. M., Lei, H. Y., Su, I. J. & Yu, C. K. (2007). Immunity to avirulent enterovirus 71 and Coxsackie A16 virus protects against enterovirus 71 infection in mice. *J Virol* **81**, 10310–10315.

Yoshikawa, T., Iwasaki, T., Ida-Hosonuma, M., Yoneyama, M., Fujita, T., Horie, H., Miyazawa, M., Abe, S., Simizu, B. & Koike, S. (2006). Role of the alpha/beta interferon response in the acquisition of susceptibility to poliovirus by kidney cells in culture. *J Virol* **80**, 4313–4325.

Yu, C. K., Chen, C. C., Chen, C. L., Wang, J. R., Liu, C. C., Yan, J. J. & Su, I. J. (2000). Neutralizing antibody provided protection against enterovirus type 71 lethal challenge in neonatal mice. *J Biomed Sci* **7**, 523–528.

Laboratory and Epidemiology Communications

Phylogenetic Analysis of Nucleoprotein (*N*) Gene of Measles Viruses Prevalent in Okinawa, Japan, during 2003 - 2007

Katsuya Taira*, Masaji Nakamura, Shou Okano, Minoru Nidaira, Jun Kudaka, Kiyomasa Itokazu, Takeyasu Taira, Toru Itokazu[1], Masao Chinen[2], Tomimasa Sunagawa[3] and Hirokazu Kimura[3]

Department of Biological Sciences, Okinawa Prefectural Institute of Health and Environment; [1]Health Promotion Division, Okinawa Prefectural Department of Health and Welfare; [2]Chinen Pediatric Clinic, Okinawa; and [3]Infectious Diseases Surveillance Center, National Institute of Infectious Diseases, Tokyo, Japan

Communicated by Masato Tashiro

(Accepted March 7, 2008)

Outbreaks of measles still occur every 5 to 7 years in Japan (1,2). In Okinawa Prefecture, two large measles outbreaks occurred during 1998 - 2001 (3), and both were attributed to the population's low immunity level (about 60 to 70%). Unfortunately, these outbreaks resulted in the deaths of 9 children. Therefore, to achieve a measles-free population, the Okinawa Prefecture Office and the Okinawa Medical Association have aggressively promoted measles immunizations, and to date have covered more than 95% of the children in the prefecture. In addition, to find all patients with measles, the prefecture office enforced sentinel surveillance systems, including laboratory confirmation of measles. As a result, the number of patients with measles rapidly decreased in the prefecture. However, a small number of patients with measles, fewer than 20, have been reported. To better understand the molecular epidemiology of the recently prevalent measles viruses (MVs), we performed sequence and phylogenetic analyses of their nucleoprotein (*N*) genes.

Throat swabs and blood samples were collected and served as test specimens in this study. All measles patients develop typical clinical symptoms: high fever, cough, conjunctivitis, Koplik's spots on the buccal mucosa, and a rash initially on the face, torso, upper neck, and back, spreading eventually to the hands and feet. Virus RNA was extracted from the samples using a QIAamp Viral RNA Mini kit (Qiagen, Valencia, Calif., USA) and suspended in DNase/RNase-free water. Following RNA extraction, reverse transcriptase-polymerase chain reaction (PCR) and nested PCR were performed as previously described (4). Amplicons were purified using a QIAquick PCR Purification kit (Qiagen), and nucleotide sequences were determined by direct sequencing (4). The nucleotide sequences of the partial *N* gene of MVs (385 bp) were analyzed phylogenetically using Molecular Evolutionary Genetics Analysis (MEGA) software version 4 (5). Evolutionary distances were estimated using Kimura's two-parameter method, and phylogenetic trees were constructed using the neighbor-

*Corresponding author: Mailing address: Department of Biological Sciences, Okinawa Prefectural Institute of Health and Environment, 2085 Ozato, Nanjo, Okinawa 910-1202, Japan. Tel: +81-98-945-0785, Fax: +81-98-945-9366, E-mail: tairktsy@pref.okinawa.lg.jp

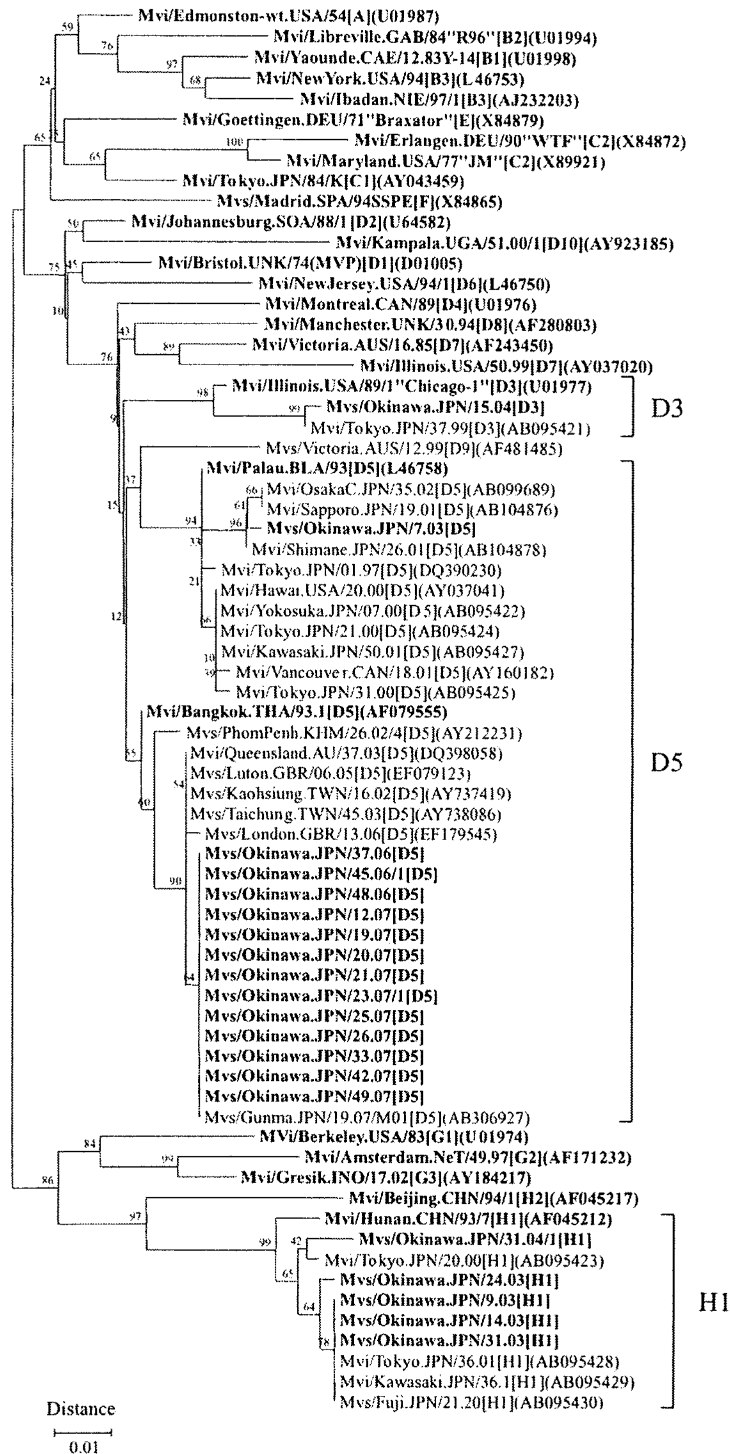

Fig. 1. Phylogenetic tree constructed based on the nucleotide protein (*N*) gene sequences of various strains of the measles virus. Evolutionary distance was calculated using Kimura's two-parameter method, and the tree was plotted using the neighbor-joining method. Numbers at each branch indicate the bootstrap values of the clusters supported by that branch. Numbers in parentheses are GenBank accession numbers. Genotypes of the reference and present strains are indicated in bold.

joining (NJ) method (6). Each tree's reliability was estimated using 1,000 bootstrap replications.

Table 1 summarizes the cases, MV genotypes, and epidemiologic data. Of 20 MV strains, 1, 14, and 5 strains had genotypes D3, D5, and H1, respectively. The strains detected in 2003 had genotypes D5 (1 strain) and H1 (4 strains), and those detected in 2004 had genotypes D3 (1 strain) and H1 (1 strain). In 2005, no MV strains were detected because no patients with measles were reported in the surveillance project. The 13 MV strains detected in 2006 and 2007 had only genotype D5. However, 15 cases were sporadic in the two outbreaks, each of which involved more than 10 patients. Epi-

Table 1. Summary of epidemiologic and measles virus data

Case no.	Month, year	Activity[1]	Epidemiology-based infection source	Virus	Genotype
1	Feb, 2003	Sporadic case	Unknown	MVs/Okinawa.JPN/7.03	D5
2	Feb, 2003	Sporadic case	Unknown	MVs/Okinawa.JPN/9.03	H1
3	Apr, 2003	Sporadic case	Unknown	MVs/Okinawa.JPN/14.03	H1
4	Jun, 2003	Sporadic case	Kanto area	MVs/Okinawa.JPN/24.03	H1
5	Jul, 2003	Sporadic case	Unknown	MVs/Okinawa.JPN/31.03	H1
6	Apr, 2004	Outbreak: 5 cases	Unknown	MVs/Okinawa.JPN/15.04	D3
7	Jul, 2004	Outbreak: 8 cases	Unknown	MVs/Okinawa.JPN/31.04/1	H1
8	Sep, 2006	Outbreak: 12 cases	Kanto area	MVs/Okinawa.JPN/37.06	D5
9	Nov, 2006	Outbreak: 4 cases	Kanto area	MVs/Okinawa.JPN/45.06/1	D5
10	Dec, 2006	Sporadic case (1)	Kanto area	MVs/Okinawa.JPN/48.06	D5
11	Mar, 2007	Sporadic case	Kanto area	MVs/Okinawa.JPN/12.07	D5
12	May, 2007	Sporadic case	Kanto area	MVs/Okinawa.JPN/19.07	D5
13	May, 2007	Sporadic case (1)	Kyushu area	MVs/Okinawa.JPN/20.07	D5
14	May, 2007	Sporadic case (1)	Kanto area	MVs/Okinawa.JPN/21.07	D5
15	Jul, 2007	Sporadic case	Kanto area	MVs/Okinawa.JPN/23.07/1	D5
16	Jul, 2007	Sporadic case	Kanto area	MVs/Okinawa.JPN/25.07	D5
17	Jul, 2007	Sporadic case	Kanto or Shikoku area	MVs/Okinawa.JPN/26.07	D5
18	Aug, 2007	Sporadic case	Kanto area	MVs/Okinawa.JPN/33.07	D5
19	Oct, 2007	Outbreak: 10 cases	Kanto area	MVs/Okinawa.JPN/42.07	D5
20	Dec, 2007	Sporadic case	Kyushu area	MVs/Okinawa.JPN/49.07	D5

[1]: For sporadic case, the number of cases that spread is indicated in parentheses.

demiologic investigation showed that most of the patients were high school students and adults who had traveled to other areas of Japan (Kanto, Shikoku, or Kyusyu). Thus, most of the patients might have been infected in areas outside Okinawa Prefecture. We constructed a phylogenetic tree based on the *N* gene of the presently isolated MV strains, including reference strains (Figure 1). All MV genotypes (D3, D5, and H1) were located in the same cluster as the strains detected from other areas of Japan (Sapporo, Gunma, Tokyo, Osaka, and Shimane). In particular, the present strains with genotype D5 were highly homologous. These results suggest that the present strains and other local strains were closely related genetically. Moreover, various MVs with the D5 genotype detected from other areas of Japan in 2007 (Gunma Prefecture and the Tokyo metropolitan area) were highly homologous (7).

Okinawa Prefecture is located in a subtropical area and consists of hundreds of Ryukyu Islands with many famous sightseeing spots; the prefecture draws approximately 6 million visitors per year. At present, only a small number of the prefecture's population may be susceptible to measles because of the regular and widespread measles immunization program. However, since the disease is highly contagious in humans (8,9), up-to-date information on the epidemic status of measles in the prefecture and in Japan as a whole is needed because of its rapid spread from one area to another.

This article appeared in part in the Infectious Agents Surveillance Report (IASR), vol. 28, p. 145-147 and p. 245-247, 2007 in Japanese.

REFERENCES

1. Okafuji, T., Okafuji, T., Fujino, M., et al. (2006): Current status of measles in Japan: molecular and seroepidemiological studies. J. Infect. Chemother., 12, 343-348.
2. Nakayama, T., Zhou, J. and Fujino, M. (2003): Current status of measles in Japan. J. Infect. Chemother., 9, 1-7.
3. Okinawa Prefecture Office (2001): Annual Reports of Surveillance in Okinawa Prefecture, 82-85 (in Japanese).
4. DiStefano, D.J., Kraiouchkine, N., Mallette, L., et al. (2005): Novel rotavirus VP7 typing assay using a one-step reverse transcriptase PCR protocol and product sequencing and utility of the assay for epidemiological studies and strain characterization, including serotype subgroup analysis. J. Clin. Microbiol., 43, 5876-5880.
5. Tamura, K., Dudley, J., Nei, M., et al. (2007): Molecular Evolutionary Genetics Analysis (MEGA) Software version 4.0. Mol. Biol. Evol., 24, 1596-1599.
6. Saitou, N. and Nei, M. (1987): The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol., 4, 406-425.
7. Morita, Y., Suzuki, T., Shiono, M., et al. (2007): Sequence and phylogenetic analysis of the nucleoprotein (*N*) gene in measles viruses prevalent in Gunma, Japan, in 2007. Jpn. J. Infect. Dis., 60, 402-404.
8. Griffin, D.E. (2001): Measles Virus. 4th ed. p. 1401-1441. *In* D.M. Knipe and P. M. Howley (ed.). Lippincott Williams & Wilkins, Philadelphia, USA.
9. World Health Organization (2006): Progress in reducing global measles deaths: 1999-2004. Wkly. Epidemiol. Rec., 81, 90-94.

Jpn. J. Infect. Dis., 61, 196-201, 2008

Original Article

Analysis of Monthly Isolation of Respiratory Viruses from Children by Cell Culture Using a Microplate Method: a Two-Year Study from 2004 to 2005 in Yamagata, Japan

Katsumi Mizuta*, Chieko Abiko, Yoko Aoki, Asuka Suto, Hitoshi Hoshina, Tsutomu Itagaki[1], Noriko Katsushima[2], Yoko Matsuzaki[3], Seiji Hongo[3], Masahiro Noda[4], Hirokazu Kimura[5] and Katsumi Ootani

Department of Microbiology, Yamagata Prefectural Institute of Public Health, Yamagata 990-0031; [1]Yamanobe Pediatric Clinic, Yamagata 990-0301; [2]Katsushima Pediatric Clinic, Yamagata 990-2461; [3]Department of Infectious Diseases, Yamagata University Faculty of Medicine, Yamagata 990-9585; and [4]Department of Virology III and [5]Infectious Disease Surveillance Center, National Institute of Infectious Diseases, Tokyo 208-0011, Japan

(Received October 15, 2007. Accepted March 17, 2008)

SUMMARY: Although well over 200 viral agents have been implicated in acute respiratory infections (ARIs) among children, no system able to detect such a wide range of viruses has been established. Between January 2004 and December 2005, a modified microplate method, including HEF, HEp-2, Vero E6, MDCK, RD-18S, and GMK cell lines (HHVe6MRG plate), was adopted to isolate viruses. A total of 1,551 viruses were isolated, representing both outbreaks and sporadic cases, from 4,107 nasopharyngeal specimens, at monthly isolation rates of 22.3 to 52.6%. Influenza, parainfluenza, respiratory syncytial (RS), and mumps viruses, and human metapneumovirus, enterovirus, parechovirus, rhinovirus, adenovirus, herpesvirus, and cytomegalovirus were all isolated. The use of multiple cell lines increased the isolation rates of most of these viruses. The findings showed that ARIs due to a number of respiratory viruses occurred across all seasons in succession and/or concurrently in children in the community. These data will help clinicians determine in which seasons and for which age groups they should use the rapid diagnostic test kits available for influenza virus, RS virus, and adenovirus. In conclusion, we verified that the modified microplate method was able to clarify the etiology and epidemiology of numerous viruses isolated from children with ARI.

INTRODUCTION

Up to 90% of the causal agents of acute respiratory infections (ARIs) are thought to be nonbacterial, making ARI an almost exclusively viral disease (1,2). Well over 200 viral agents have been implicated in ARI (1), but no community-based viral etiological study has been reported (3). To undertake such a study, an efficient viral detection system is necessary to clarify the etiology and epidemiology of ARIs. Currently, viral culture, antigen detection, and polymerase chain reaction (PCR) are used to detect viruses, with each method having its individual advantages and disadvantages (4). In particular, molecular methods, including PCR, reverse transcription (RT)-PCR, and real-time PCR have been considered valuable in the diagnosis of viral infections, as these methods are more rapid and sensitive than traditional antigen detection and virus isolation techniques (5-11). Although the benefits of modern molecular technologies are obvious, the simple question remains as to whether the traditional tissue culture method is still of value.

To isolate a wide range of cultivable viruses efficiently, in the mid-1980s Numazaki et al. developed a microplate method that was simpler and cheaper than the traditional tube method (12). After various modifications, this method has been used to help clarify the etiology and epidemiology of ARI in children (13-15). The original microplate method using 96-well tissue culture plates included four cell lines–the human embryonic lung fibroblast (HEF), human laryngeal carcinoma (HEp-2), African green monkey kidney (Vero), and Madin Darby Canine Kidney (MDCK)–and was named the HHVM plate, based on the first letter of each cell line (12). This system was able to isolate influenza A H3N2 (FluAH3), influenza A H1N1 (FluAH1), influenza B (FluB), RS virus, parainfluenza virus (Para) 1-3, mumps virus, adenovirus (Ad) 1-6, poliovirus (Polio) 1-3, coxackievirus (Cox) A16, CoxB1-5, echovirus (Echo) 3, 6, 7, 9, 11, 21, 22, herpes simplex virus (HSV), and cytomegalovirus (CMV) (12). Between 1988 and 1998, we also isolated influenza C (FluC), rhinovirus, Ad7, CoxA7, 9, Echo15, 18, 25, 30, and enterovirus 71 (16). However, this system demonstrated low sensitivity to CoxA viruses, except CoxA16, and insufficient sensitivity to enteroviruses (12,17). Thus, in 2001 we added two cell lines, rhabdomyosarcoma (RD-18S) and green monkey kidney (GMK), to the four original cell lines to allow for the more efficient isolation of enteroviruses (HHVMRG plate) (17). Furthermore, we discovered that human metapneumovirus (hMPV), which was first isolated from patients with ARI in the Netherlands in 2001 and had hitherto been difficult to recover using the tissue culture method, could be replicated in Vero E6 cells (18,19). We therefore substituted the Vero E6 cell line for the regular Vero cell line in 2004 to create the HHVe6MRG plate for the isolation

*Corresponding author: Mailing address: Department of Microbiology, Yamagata Prefectural Institute of Public Health, Tokamachi 1-6-6, Yamagata, Yamagata 990-0031, Japan. Tel: +81-23-627-1109, Fax: +81-23-641-7486, E-mail: mizutak@pref.yamagata.jp

of hMPV (18).

Herein, we describe the results of virus isolation from patients with ARI when this HHVe6MRG plate method was used in Yamagata, Japan, between 2004 and 2005.

MATERIALS AND METHODS

Preparation of the HHVe6MRG plate: As mentioned above, we have used the HHVe6MRG plate, including the HEF, HEp-2, Vero E6, MDCK, RD-18S, and GMK cell lines, since January, 2004. Two rows of each cell line were prepared in 96-well tissue culture plates (Greiner Bio-One, Frickenhausen, Germany). The HEF cell line, which was originally deposited by I. Ishikawa, was purchased from Riken Cell Bank, Tsukuba, Japan. HEp-2 and MDCK cells were obtained from Dr. H. Nishimura, Virus Research Center, Clinical Research Division, National Hospital Organization, Sendai Medical Center, Sendai, Japan. Vero E6 cells were provided by the National Institute of Infectious Diseases, Tokyo, Japan, and the RD-18S cell line was a gift from Dr. T. Fujimoto, Hyogo Prefectural Institute of Public Health and Environmental Sciences, Kobe, Hyogo, Japan. We also used the GMK cell line, which was being stored in our laboratory, though its origin was unclear.

The composition of the growth medium (GM) and maintenance medium (MM) were slightly modified from those used in previous reports (12,17). As shown in Table 1, fetal bovine serum (Invitrogen Co., Carlsbad, Calif., USA), calf serum (Thermo Electron Co., Melbourne, Australia), vitamin solution (100× concentrate; Sanko Junyaku, Tokyo, Japan), and crystallized trypsin (T-8003; Sigma, St. Louis, Mo., USA), were added to Eagle's minimum essential medium (MEM, Nissui No3; Nissui Pharmaceutical Co., Tokyo, Japan), respectively. When the monolayer of each cell line was ready for specimen inoculation, the plates were washed with phosphate-buffered saline without calcium or magnesium, and 100 μl of MM was added to each well.

Virus isolation and identification: Between January 2004 and December 2005, 4,107 throat and nasal swab specimens were collected from patients at pediatric clinics working in collaboration with the Yamagata Prefectural health authorities as part of the national surveillance of viral diseases in Japan. Patients were clinically diagnosed as having ARI with fever and/or cough and/or rhinorrhea. Among these specimens, 3,984 (97.0%) were from patients ≤ 15 years old, 111 (2.7%) were from patients >15, and 12 (0.3%) were from patients of unknown age.

To isolate viruses, specimens were first placed in tubes containing 3 ml of transport medium consisting of MEM with 0.5% gelatin, 100 units of penicillin, and 100 μg of streptomycin per ml. They were then transported to the Department of Microbiology, Yamagata Prefectural Institute of Public Health, Yamagata. After centrifugation of the specimens at 3,000 rpm for 15 min, 75 μl of the supernatant was inoculated directly onto the two wells of each cell line. The remainder of each specimen was stored at −80°C. The inoculated plates were centrifuged for 20 min at 2,000 rpm, incubated at 33°C in a 5% CO_2 incubator, and assessed for cytopathic effect (CPE) for 14 days, except for the Vero E6 cell lines, which were observed for approximately 1 month without medium change in order to isolate hMPVs (18).

Cultures were tested for hemagglutination (HA) on the 7th - 8th days after specimen inoculation for all specimens, as follows. Fifty microliters of MDCK primary culture fluid were transferred to two wells of a 96-well U-shaped plate (Nalge Nunc, International K.K., Tokyo, Japan) and an equal volume of guinea pig erythrocytes (0.8%) or chicken erythrocytes (0.5%) was added to each well. After vibration, the plates were incubated at 4°C for 45 min, and then the HA positivity for each erythrocyte was examined. HA testing was also performed as necessary for the Vero E6 primary culture fluid.

When a CPE was observed, the whole well culture was transferred directly into one well of the identical cell line, which was prepared in advance in 24-well culture plates (Greiner Bio-One). As most viruses afford typical microscopic CPE images and cell sensitivity patterns, we speculated that they could be identified from the observed CPE. When cultures were found to be HA-positive regardless of any clear CPE, we proceeded with the identification step as previously described (12,17). However, when viruses could not be identified using these methods, we also used an RT-PCR method and sequencing analyses.

RESULTS

Using HHVe6MRG plates, 1,551 viruses were isolated from the 4,107 throat and nasal swabs at a virus isolation rate of 37.8%. Two kinds of viruses were isolated from 8 and 15 identical patients in 2004 and in 2005, respectively (dual infection).

The number of isolates cultivated in each cell line is shown by virus type in Table 2. All influenza viruses, including AH1, AH3, B, and C, were recovered using the MDCK cell line. Parainfluenza viruses were propagated in the Vero E6 and MDCK cell lines, though Para1 replicated more efficiently in the Vero E6 cells, whereas Para3 replicated more readily in the MDCK cells. Para2, along with hMPVs, was only isolated in the Vero E6 cells. The respiratory syncytial (RS) virus grew and showed optimum syncytia formation in the HEp-2 cells, although syncytia were observed in other cells. Forty-one and 31 mumps viruses clearly showed large syncytia in the GMK and Vero E6 cells, respectively. Enteroviruses

Table 1. Composition of growth and maintenance medium for the six cell lines of the HHVe6MRG plate

Cell line	HEF	HEp-2	Vero E6	MDCK	RD-18S	GMK
Growth medium	MEM[1]+10%[2]FBS[3]	MEM+5%CS[4]+ 1.7%glucose	MEM+10%FBS	MEM+5%FBS+5%CS	MEM+8%FBS	MEM+5%FBS+ 5%CS
Maintenance medium	MEM+2%FBS	MEM+2%FBS	MEM+0.2%glucose+5% vitamine solution+crystalized trypsin (2 μg/ml)[5]	MEM+0.2%glucose+5% vitamine solution+crystalized trypsin (3.5 μg/ml)	MEM+2%FBS	MEM+2%FBS

[1]: Eagle's minimum essential medium (MEM) including antibiotics (penicillin 50 units/ml and streptomycin 0.4 mg/ml).
[2,5]: Percentage and trypsin density indicate final concentration.
[3]: Fetal bovine serum.
[4]: Calf serum.

Table 2. Number of viruses in each cell line from children with ARI by the HHVe6MRG microplate method

Virus		Total	Cell line					
			HEF	HEp-2	Vero E6	MDCK	RD-18S	GMK
Flu	AH1	2				2[1)]		
	AH3	244				244		
	B	107				107		
	C	28				28		
Para	1	89			71	23		
	2	41			41			
	3	115			23	101		
	4	1	1					
hMPV		79			79			
RS		109	35	84	17		2	3
Mumps		43		2	31			41
Entero	CoxA2	29					29	
	CoxA4	55					55	
	CoxA6	31					31	
	CoxA14	2	1					2
	CoxA16	7			4		1	7
	CoxB1	41		25	16			39
	CoxB3	17	2	12	11			12
	CoxB4	5		2	2			5
	CoxB5	21	1	17	10			16
	Echo3	34	32				28	
	Echo7	46	39		9		38	6
	Echo16	21	13				20	
	Polio1	6	4	4	5		6	4
	Polio2	5	2	2	4		5	3
	Untyped	2	1		2		1	1
Parecho		9		5	3			3
Rhino		21	21					
Ad	1	64	53	42	44		4	15
	2	64	53	53	42		6	14
	3	80	74	34	38		4	5
	4	1	1	1				
	5	18	12	13	13		3	6
	6	2	2					1
	Untyped	4	1		3			
HSV		46	44	25	31		18	29
CMV		62	62					

[1)]: Dual infection cases were counted independently for each virus.

were isolated using all cell lines except for MDCK, whereas rhinoviruses could be cultivated only in HEF cells. Most adenoviruses could be propagated in HEF, HEp-2, and Vero E6 cells, but Ad3 grew best in HEF cells. HSVs showed CPEs in all cell lines except for MDCK, and CMV growth was observed only in HEF cells.

The monthly distribution of the recovered viruses is shown in Tables 3 and 4. Using the HHVe6MRG plate, a number of respiratory viruses were isolated from ARI patients across all seasons, covering outbreaks as well as sporadic cases. Viruses were isolated each month throughout the 2-year study period at an isolation rate of between 22.3 and 52.6%.

We then examined differences in virus types among the different age groups. Patients were divided into four age groups: 0 - 1 year, 2 - 4 years, 5 - 11 years, and >11 years. The number of isolates for each virus is shown in Table 5. The isolation rate was highest in the 0 - 1 year age group (42.4%) and gradually decreased with age. For patients aged 0 - 1 year, Para3, RS virus, Ad1, Ad2, and CMV were frequently isolated. For patients aged 2 - 4 years, FluAH3, Para1, RS virus, and CoxA were often isolated, whereas for patients aged 5 - 11 years, FluAH3, FluB, and CoxB were the most common isolates.

DISCUSSION

Recognizing the value of the original microplate method, we developed a modified method as described previously (17,18). The results obtained in 2004 and 2005 supported the notion that the use of multiple cell lines can increase the isolation rate of most viruses (Table 2). For example, although most RS viruses (84/109) were isolated using the HEp-2 cell line, 25 strains were isolated by cell lines other than HEp-2, and the use of GMK cell lines increased the virus isolation rate of mumps, CoxA16, CoxB1, and CoxB4. Furthermore, HA testing with chicken erythrocytes for the primary MDCK culture fluid provided a simple and effective way of differentiating FluC from FluA and FluB, as FluC is known to agglutinate chicken but not guinea pig erythrocytes.

Although the data for several viruses have been published independently elsewhere, we here report the complete virus isolation data for a 2-year period in the local community of Yamagata. Detection data of such a wide range of viruses have rarely been reported, except in several studies that recently tried to detect over 10 types of respiratory viruses using an RT-PCR method (5,20). Together, Tables 3 and 4 clearly show that ARIs due to many respiratory viruses occurred in succession and/or concurrently in children in the

Table 3. Monthly distribution of viruses isolated from children with ARI by the HHVe6MRG microplate method (2004)

Virus		Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total
Flu	AH1													0
	AH3	36	30	17	6									89
	B				3						2			5
	C				10	15								25
Para	1				1	1	3	2	1		2			10
	2				1		1	3		14	9	9	4	41
	3					5	30	7	1					43
	4											1		1
hMPV			2	6	10									18
RS		2							5	12	12	22	6	59
Mumps		1					2	1				1	2	7
Entero	CoxA2							1	8	15	4	1		29
	CoxA4					5	20	25	4	1				55
	CoxA6													0
	CoxA14													0
	CoxA16											1		1
	CoxB1	4	6		5	8	5	11	1	1				41
	CoxB3										2	1		3
	CoxB4								1					1
	CoxB5		1											1
	Echo3						2	14	4	11	1	1	1	34
	Echo7	7	10				8	6	1	8	4	1	1	46
	Echo16													0
	Polio1 - 3			2	1						3			6
Parecho							1			3	2			6
Rhino		1		1	2	1	1	1	1	1		1		10
Ad	1	1		1		2	5	3			1	1	2	16
	2		4	1	1	2	6	6		1	1	2	9	33
	3	5	7	3	3	1	1					3		23
	4												1	1
	5	1	1	1	2	1	3	1					2	12
	6	1												1
HSV		2		3	2	2		3	1	2	1	1		17
CMV		1	3		2	2	5	4	3	2	3	3	4	32
Untyped											1		2	3
Total		62	64	35	49	45	93	88	31	71	48	49	34	669
No. tested		125	163	157	118	131	216	178	120	207	145	160	143	1,863[1]
Isolation rate (%)		49.6	39.3	22.3	41.5	34.4	43.1	49.4	25.8	34.3	33.1	30.6	23.8	35.9

[1]: Five measles-suspected cases were excluded.

community. Applying the microplate method throughout the year enabled us to isolate viruses that circulated at unexpected times, contrary to their described epidemiology. Although Williams et al. showed the epidemiologic patterns of lower respiratory tract infections with hMPV, RS virus, parainfluenza virus, and other viruses (21), our data suggested that all of these viruses should be considered causative agents even in the summer. The observation that Para1, Para3, hMPV, RS virus, and rhinoviruses circulated between April and September 2005 is quite important in that ARIs due to these viruses show similar symptoms, including lower respiratory infections, bronchitis and pneumonia (3,19,22), and the etiological diagnosis of these viruses is difficult. These data will help pediatricians determine in which seasons they should use the rapid diagnostic test kits available for influenza virus, RS virus, and adenovirus. Moreover, information on which viruses are commonly detected in the various age groups is sure to aid diagnosis and treatment.

Once isolated, the stocked isolates are available at any time for further epidemiological analyses. This affords a great advantage over RT-PCR methods. For example, to date we have clarified the molecular epidemiology and performed antigenic analyses on adenovirus, FluB, FluC, EV71, and hMPV (18,23-28), utilizing stocked isolates. Further, as infectious diseases can spread worldwide rapidly, epidemiological surveillance in a local community such as Yamagata now has the potential to act as part of a national, regional, or even international surveillance of emerging and re-emerging diseases. Indeed, a number of studies have already been produced from the results of microplate systems (29-31). Thus, we believe that a virus isolation technique such as a modified microplate system is still of great use in clarifying the etiology and epidemiology of ARI in children, even though RT-PCR methods are superior in terms of rapidity and sensitivity, especially for clinical purposes (3,5-10).

As we believe that virus isolation is still an important basic method for clarifying the etiology and epidemiology of ARIs, our aim is to further modify the microplate system described herein in order to better contribute to virus detection and epidemiological analysis systems in the future.

ACKNOWLEDGMENTS

We thank the doctors, nurses and people of Yamagata Prefecture for their assistance and collaboration in the surveillance of viral infectious diseases. We also thank Dr. H. Nishimura, Virus Research Center, Clinical Research Division, National Hospital Organization, Sendai Medical Center, Sendai, Japan and Dr. T. Fujimoto, Hyogo Prefectural Institute of Public Health and Environmental Sciences, Kobe, Hyogo, Japan for providing us with cell lines

Table 4. Monthly distribution of viruses isolated from children with ARI by the HHVe6MRG microplate method (2005)

Virus		Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total
Flu	AH1		1	1										2
	AH3	6	41	49	10	10		2	1			15	21	155
	B	9	50	31	9	3								102
	C						1	1		1				3
Para	1				5	15	14	16	13	4	3	4	5	79
	2													0
	3					4	22	29	14	3				72
	4													0
hMPV				1	10	15	12	9	10	1		2	1	61
RS		6	1	4		4	1	1	1	2	5	11	14	50
Mumps			3	2	2	3	5	1	5		5	7	3	36
Entero	CoxA2													0
	CoxA4													0
	CoxA6			1	3	9	11	5	2					31
	CoxA14	1		1										2
	CoxA16									1	3	2		6
	CoxB1													0
	CoxB3							2	6	3		2	1	14
	CoxB4									1	2		1	4
	CoxB5							1	3	9	4	2	1	20
	Echo3													0
	Echo7													0
	Echo16						2		7	5		1	6	21
	Polio1-3			2		2					1			5
Parecho									1	2				3
Rhino					1	3	2			1	3	1		11
Ad	1	5	4		5	8	7	3	7		3	4	2	48
	2	4	3		3	3	3	1	4	1		5	4	31
	3	5	1	1			1	6	15	7	3	7	11	57
	4													0
	5			1			1	2	1				1	6
	6									1				1
HSV		5	3	2	1	1	1	1	2	3	4	5	1	29
CMV		1	4			2		7	3	1	4	6	2	30
Untyped							1		2					3
Total		42	111	96	49	82	84	87	97	46	40	74	74	882
No. tested		124	211	209	168	202	223	211	200	176	160	185	175	2,244
Isolation rate (%)		33.9	52.6	45.9	29.2	40.6	37.7	41.2	48.5	26.1	25	40	42.3	39.3

used in this study.

This study was partially supported by Research on Emerging and Re-emerging Infectious Diseases, Labour and Welfare programs from the Ministry of Health, Labour and Welfare of Japan.

REFERENCES

1. Hodes, D.S. (1998): Respiratory infections and sinusitis. p. 362-401. *In* Katz, S.L., Gershon, A.A., Hotez, P.J., (ed.), Infectious Diseases of Children. 10th ed. Mosby-Year Book, Missouri.
2. Mäkelä, M.J., Puhakka, T., Ruuskanen, O., et al. (1998): Viruses and bacteria in the etiology of the common cold. J. Clin. Microbiol., 36, 539-542.
3. Kusel, M.M.H., De Klerk, N.H., Holt, P.G., et al. (2006): Role of respiratory viruses in acute upper and lower respiratory tract illness in the first year of life. A Birth Cohort Study. Pediatr. Infect. Dis. J., 25, 680-686.
4. Heikkinen, T. and Järvinen, A. (2003): The common cold. Lancet, 361, 51-59.
5. Bellau-Pujol, S., Vabret, A., Legrand, L., et al. (2005): Development of three multiplex RT-PCR assays for the detection of 12 respiratory RNA viruses. J. Virol. Methods, 126, 53-63.
6. Echevarría, J.E., Erdman, D.D., Swierkosz, E.M., et al. (1998): Simultaneous detection and identification of human parainfluenza viruses 1, 2, and 3 from clinical samples by multiplex PCR. J. Clin. Microbiol., 36, 1388-1391.
7. Fan, J., Henrickson, K.J. and Savatski, L.L. (1998): Rapid simultaneous diagnosis of infections with respiratory syncytial viruses A and B, influenza viruses A and B, and human parainfluenza virus types 1, 2, and 3, by multiplex quantitative reverse transcription-polymerase chain reaction-enzyme hybridization assay (Hexaplex). Clin. Infect. Dis., 26, 1397-1402.
8. Osiowy, C. (1998): Direct detection of respiratory syncytial virus, parainfluenza virus, and adenovirus in clinical respiratory specimens by a multiplex reverse transcription-PCR assay. J. Clin. Microbiol., 36, 3149-3154.
9. Syrmis, M.W., Whiley, D.M., Thomas, M., et al. (2004): A sensitive, specific, and cost-effective multiplex reverse transcriptase-PCR assay for the detection of seven common respiratory viruses in respiratory samples. J. Mol. Diagn., 6, 125-131.
10. Templeton, K.E., Scheltinga, S.A., Beersma, M.F.C., et al. (2004): Rapid and sensitive method using multiplex real-time PCR for diagnosis of infections by influenza A and influenza B viruses, respiratory syncytial virus, and parainfluenza viruses 1, 2, 3, and 4. J. Clin. Microbiol., 42, 1564-1569.
11. Weinberg, G.A., Erdman, D.D., Edwards, K.M., et al. (2004): Superiority of reverse-transcription polymerase chain reaction to conventional viral culture in the diagnosis of acute respiratory tract infections in children. J. Infect. Dis., 189, 706-710.
12. Numazaki, Y., Oshima, T., Ohmi, A., et al. (1987): A microplate method for isolation of viruses from infants and children with acute respiratory infections. Microbiol. Immunol., 31, 1085-1095.
13. Brumback, B.G., Cunningham, D.M., Morris, M.V., et al. (1995): Rapid culture for influenza virus, types A and B, in 96-well plates. Clin. Diagn. Virol., 4, 251-256.
14. Henrickson, K.J., Kuhn, S.M., Savatski, L.L., et al. (1994): Recovery of human parainfluenza virus types one and two. J. Virol. Methods, 46, 189-206.
15. Jennings, L.C., Anderson, T.P., Werno, A.M., et al. (2004): Viral etiology of acute respiratory tract infections in children presenting to hospital. Pediatr. Infect. Dis. J., 23, 1003-1007.
16. Mizuta, K., Katsushima, N., Sakamoto, M., et al. (2000): Epidemiology

Table 5. Number of isolates and virus isolation rate among different age groups based on the HHVe6MRG microplate method in Yamagata, Japan, between 2004 and 2005

Virus		No. (%) of isolates in age group of:			
		0 - 1 year	2 - 4 years	5 - 11 years	>11 years
Flu	AH1	0	0	2 (0.2)	0
	AH3	20 (1.7)	60 (4.3)	125 (10.3)	39 (13.8)
	B	12 (1.0)	31 (2.2)	54 (4.5)	10 (3.5)
	C	11 (0.9)	12 (0.9)	5 (0.4)	0
Para	1	23 (1.9)	42 (3.0)	16 (1.3)	7 (2.5)
	2	11 (0.9)	21 (1.5)	8 (0.7)	1 (0.4)
	3	63 (5.2)	39 (2.8)	9 (0.7)	4 (1.4)
	4	0	1 (0.07)	0	0
hMPV		25 (2.1)	39 (2.8)	13 (1.1)	2 (0.7)
RS		50 (4.1)	51 (3.7)	8 (0.7)	0
Mumps		12 (1.0)	11 (0.8)	18 (1.5)	2 (0.7)
CoxA		35 (2.9)	61 (4.4)	26 (2.1)	2 (0.7)
CoxB		18 (1.5)	25 (1.8)	37 (3.1)	3 (1.1)
Echo		22 (1.8)	38 (2.7)	35 (2.9)	6 (2.1)
Polio		8 (0.7)	2 (0.1)	0	0
Parecho		7 (0.6)	2 (0.1)	0	0
Rhino		6 (0.5)	6 (0.4)	7 (0.6)	2 (0.7)
Ad	1	39 (3.2)	22 (1.6)	3 (0.2)	0
	2	38 (3.2)	19 (1.4)	7 (0.6)	0
	3	22 (1.8)	31 (2.2)	24 (2.0)	2 (0.7)
	4	1 (0.08)	0	0	0
	5	12 (1.0)	3 (0.2)	3 (0.2)	0
	6	0	1 (0.07)	1 (0.08)	0
HSV		16 (1.3)	21 (1.5)	6 (0.5)	3 (1.1)
CMV		57 (4.7)	4 (0.3)	0	0
Untyped		3	1	2	0
Total no. of specimens		1,205	1,395	1,213	282
Total no. of isolates		511	543	409	83
Isolation rate (%)		42.4	38.9	33.7	29.4

of viral acute respiratory infections (ARI) among children in Yamagata (1988 - 1998). Bull.Yamagata Med. Assoc., 589, 25-34 (in Japanese).

17. Mizuta, K., Abiko, C., Goto, H., et al. (2003): Enterovirus isolation from children with acute respiratory infections and presumptive identification by a modified microplate method. Int. J. Infect. Dis., 7, 138-142.
18. Abiko, C., Mizuta, K., Itagaki, T., et al. (2007): Outbreak of human metapneumovirus detected by use of the Vero E6 cell line in isolates collected in Yamagata, Japan, in 2004 and 2005. J. Clin. Microbiol., 45, 1912-1919.
19. Van den Hoogen, B.G., De Jong, J.C., Groen, J., et al. (2001): A newly discovered human pneumovirus isolated from young children with respiratory tract disease. Nat. Med., 7, 719-724.
20. Lam, W.Y., Yeung, A.C.M., Tang, J.W., et al. (2007): Rapid multiplex nested PCR for detection of respiratory viruses. J. Clin. Microbiol., 45, 3631-3640.
21. Williams, J.V., Harris, P.A., Tollefson, S.J., et al. (2004): Human metapneumovirus and lower respiratory tract disease in otherwise healthy infants and children. N. Engl. J. Med., 350, 443-450.
22. Isaacs, D. (1996): Clinical spectrum of disease in children. p.47-60. *In* Myint, S. and Taylor-Robinson, D. (ed.), Viral and Other Infections of the Human Respiratory Tract. 1st ed. Chapman & Hall, London.
23. Matsuzaki, Y., Abiko, C., Mizuta, K., et al. (2007): A nationwide epidemic of influenza C virus infection in Japan in 2004. J. Clin. Microbiol., 45, 783-788.
24. Matsuzaki, Y., Katsushima, N., Nagai, Y., et al. (2006): Clinical features of influenza C virus infection in children. J. Infect. Dis., 193, 1229-1235.
25. Matsuzaki, Y., Sugawara, K., Takashita, E., et al. (2004): Genetic diversity of influenza B virus: the frequent reassortment and cocirculation of the genetically distinct reassortant viruses in a community. J. Med. Virol., 74, 132-140.
26. Matsuzaki, Y., Takao, S., Shimada, S., et al. (2004): Characterization of antigenically and genetically similar influenza C viruses isolated in Japan during the 1999 - 2000 season. Epidemiol. Infect., 132, 709-720.
27. Mizuta, K., Abiko, C., Murata, T., et al. (2005): Frequent importation of enterovirus 71 from surrounding countries into the local community of Yamagata, Japan between 1998 and 2003. J. Clin. Microbiol., 43, 6171-6175.
28. Mizuta, K., Suzuki, H., Ina, Y., et al. (1994): Six-year longitudinal analysis of adenovirus type 3 genome types isolated in Yamagata, Japan. J. Med. Virol., 42, 198-202.
29. Mizuta, K., Abiko, C., Aoki, Y., et al. (2006): A slow spread of adenovirus type 7 infection after its re-emergence in Yamagata, Japan, in 1995. Microbiol. Immunol., 50, 553-558.
30. Mizuta, K., Abiko, C., Murata, T., et al. (2003): Re-emergence of echovirus type 13 infections in 2002 in Yamagata, Japan. J. Infect., 47, 243-247.
31. Mizuta, K., Katsushima, N., Ito, S., et al. (2003): A rare appearance of influenza A(H1N2) as a reassortant in a community such as Yamagata where A(H1N1) and A(H3N2) co-circulate. Microbiol. Immunol., 47, 359-361.

Journal of Medical Virology 80:1084–1089 (2008)

Clinical Impact of Human Metapneumovirus Genotypes and Genotype-Specific Seroprevalence in Yamagata, Japan

Yoko Matsuzaki,[1]* Tsutomu Itagaki,[2] Chieko Abiko,[3] Yoko Aoki,[3] Asuka Suto,[3] and Katsumi Mizuta[3]
[1]*Course of Clinical Nursing, Yamagata University Faculty of Medicine, Iida-Nishi, Yamagata, Japan*
[2]*Yamanobe Pediatric Clinic, Yamanobe, Yamagata, Japan*
[3]*Yamagata Prefectural Institute of Public Health, Toka-Machi, Yamagata, Japan*

The clinical impact of human metapneumovirus (hMPV) genotypes and the relation between the hMPV genotype in circulation and genotype-specific seroprevalence are yet to be clarified. We determined the genotypes of 93 hMPV strains that were isolated between 2004 and 2006 in Yamagata, Japan, and identified 35 genotype A2, 14 genotype B1, and 44 genotype B2 isolates. Children infected with genotype A2 hMPV were significantly older than those infected with genotype B1 hMPV. Diagnosis of laryngitis was more common in children with genotype B1 hMPV infection and wheezing was more prevalent in children with genotype B1 and B2 hMPV infection than in those with genotype A2 hMPV infection. We then examined genotype-specific seroprevalence by neutralization assay. The higher seropositive rate for the B2 genotype among the children aged 1–2 years is likely to reflect the outbreak of B2 genotype strains in the previous year in this community. The low seropositive rate for the B1 genotype among children aged 1–2 years appears to be associated with a finding that more than 70% of children infected with the B1 genotype were less than 3 years old. In conclusion, we found that the different clinical characteristics of hMPV infection may be associated with hMPV genotype, and the predominant genotype during a season and the affecting age may be closely related to genotype-specific immune status within a community. ***J. Med. Virol. 80:1084–1089, 2008.***
© 2008 Wiley-Liss, Inc.

KEY WORDS: human metapneumovirus; genotype; seroprevalence; neutralization assay

INTRODUCTION

Human metapneumovirus (hMPV) is a newly discovered pathogen associated with respiratory infections ranging from upper respiratory infections to lower respiratory infections, such as bronchitis, bronchiolitis and pneumonia, and has been classified as a member of the genus *Metapneumovirus* of the subfamily *Pneumovirinae* of the family *Paramyxoviridae* [van den Hoogen et al., 2001; Boivin et al., 2002; Bastien et al., 2003; Williams et al., 2004]. Based on genetic and phylogenetic analyses, hMPV is separated into two subgroups A and B, with each subgroup divided into genotypes 1 and 2 [van den Hoogen et al., 2004]. Each season, several different hMPV genotypes cocirculate, with the predominant genotypes varying from season to season [Mackay et al., 2006; Williams et al., 2006]. Almost all children are infected with hMPV by the age of 5 years [van den Hoogen et al., 2001; Ebihara et al., 2003; Leung et al., 2005], but the relationship between reinfection and immunity is not well understood. Further, the relationship between the predominant hMPV genotype and genotype-specific seroprevalence in a community is yet to be clarified.

Respiratory syncytial virus (RSV), which belongs to the subfamily *Pneumovirinae*, is also divided into subgroups A and B, and some studies have reported that subgroup A isolates are associated with more severe illness [Walsh et al., 1997; Gilca et al., 2006]. Although a few studies have reported differences in clinical severity between hMPV subgroups A and B [Agapov et al., 2006; Vicente et al., 2006], the relation between hMPV genotypes and severity remains unclear.

© 2008 WILEY-LISS, INC.

We have no commercial or other association that might pose a conflict of interest.

Grant sponsor: Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labour and Welfare, Japan (partial support).

*Correspondence to: Yoko Matsuzaki, Course of Clinical Nursing, Yamagata University Faculty of Medicine, Iida-Nishi, Yamagata 990-9585, Japan.
E-mail: matuzaki@med.id.yamagata-u.ac.jp

Accepted 6 March 2008
DOI 10.1002/jmv.21194
Published online in Wiley InterScience
(www.interscience.wiley.com)

WILEY InterScience® DISCOVER SOMETHING GREAT

We recently found that Vero E6 cells are useful for the isolation of hMPV and reported an outbreak of hMPV in Yamagata, Japan in 2004 and 2005 [Abiko et al., 2007]. In this article, we clarify the epidemiological characteristics of the hMPV strains isolated during outbreaks in a Yamagata community between 2004 and 2006, and estimate the impact of genotype on clinical features and seroprevalence. Additionally, we report the neutralizing antibody response of children who were infected with hMPV.

MATERIALS AND METHODS

Collection of Specimens

From January 2004 to December 2006, 3,649 throat and nasal specimens were collected from children ≤15 years old who visited Yamanobe Pediatric Clinic in Yamagata with acute respiratory symptoms. After collection, the specimens were kept in tubes containing a transport medium for 1–4 days at 4°C and then transported to Yamagata Prefectural Institute of Public Health for virus isolation.

Virus Isolation and Identification

Virus isolation was carried out using a modified microplate method as described previously [Mizuta et al., 2005; Matsuzaki et al., 2006; Abiko et al., 2007]. Briefly, after centrifugation at 3,000 rpm for 15 min, 75μl of supernatant from each sample was inoculated onto the following cell lines in 96-well plates: human embryonic fibroblast (HEF), Hep-2, Vero E6, MDCK, rhabdomyosarcoma (RD-18S), and green monkey kidney cell line. The inoculated plates were then incubated at 33°C in a CO_2 incubator. When a suspected hMPV CPE was observed in the Vero E6 cell line, viral identification was carried out by RT-PCR and then determination of genotype was performed by sequence analysis for a part of fusion region using primers MPVF1f, MPV F1r, BF101, and BF104 [Peret et al., 2002; van den Hoogen et al., 2004].

Serum Samples

Human serum samples were collected in Yamagata between April and September 2005, as part of the national epidemiological surveillance of vaccine-preventable diseases led by the Japanese Ministry of Health, Labor and Welfare. We used a total of 213 samples; 14 samples from children <1 year old, 20 from 1 year olds, 17 from 2-year olds, 17 from 3-year olds, 12 from 4-year olds, 10 from 5-year olds, 31 from 6- to 10-year olds, 36 from 11- to 15-year olds, 25 from 16- to 30-year olds, and 31 from residents over the age of 30, from whom informed consent was received (either from the individual or guardian). The samples from children less than 15 years of age were all collected from children with acute respiratory symptoms or gastroenteritis at Yamanobe Pediatric Clinic. The samples were stored at −20°C until use.

Neutralization Test

Three hMPV strains 871-Yamagata-05, 901-Yamagata-05, and 1132-Yamagata-05, which are representative strains for genotypes B1, A2, and B2, respectively, were used as antigens of the neutralization test. A total of 60 μl of MEM containing serial twofold dilutions of sera, which had been inactivated at 56°C for 30 min, were incubated with an equal volume of MEM containing approximately 100 $TCID_{50}$ of the virus at 37°C for 1 hr. After incubation, 50 μl of the virus-antibody mixture was added to each of 2 wells of a 96-well plate containing Vero E6 cells, washed and then MEM containing 2 μg/ml trypsin was added. The cells were grown for 7 days at 33°C and examined for hMPV-induced cytopathic effect (CPE). The titer was expressed as the reciprocal of the serum dilution that completely inhibited CPE.

Clinical Data

Clinical data of the children from whom hMPVs were isolated were obtained retrospectively from their medical records. Children from whom another respiratory virus was isolated at the same time were not included in the analysis.

Statistical Analysis

Statistical analysis was performed using the StatView-J 4.02. Categorical variables were compared by χ^2 test, and the Mann–Whitney U-test was used to compare median values. A P-value of <0.05 was regarded as statistically significant.

RESULTS

Epidemiological Characteristics of hMPV Infection

From 1 January 2004 to 31 December 2006, 3,649 specimens were obtained from children with acute respiratory tract infections, and a total of 93 (2.5%) hMPVs were isolated. The isolation rate by year was 1.5% (18 from 1,177 specimens) in 2004, 3.7% (44 from 1,192 specimens) in 2005 and 2.4% (31 from 1,280 specimens) in 2006.

Genotypes of the isolated hMPVs were identified from the results of phylogenetic tree analysis performed using the sequenced region of the F gene. The monthly distribution and genotypes are shown in Figure 1. The number of subgroup A genotypes isolated from samples collected over 3-year period was 35 (38%) and that of subgroup B genotypes 58 (62%). Genotype A1 was not isolated during the study period; therefore, the proportions of A2, B1, and B2 genotypes were 35 (38%), 14 (15%), and 44 (47%), respectively. Roughly equivalent numbers of genotype A2 isolates were observed in 2005 (54% of total A2 isolates) and 2006 (46% of total A2 isolates), whereas genotype B1 was predominant in 2005 (93% of total B1 isolates), and genotype B2 strains were in circulation over all three seasons (39% of total B2 isolates in 2004, 27% in 2005, and 34% in 2006).

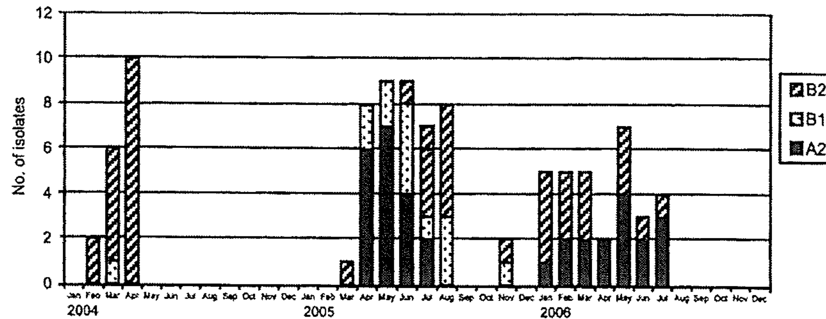

Fig. 1. Monthly distribution and genotypes of hMPV strains isolated from specimens collected at Yamanobe Pediatric Clinic in Yamagata, Japan between 2004 and 2006.

The median age of the children with hMPV infection was 2 years (range, 1–7 years) in 2004, 2 years (range, 9 months–11 years) in 2005, and 3 years (range, 7 months–13 years) in 2006.

Comparison of Clinical Characteristics Between hMPV Genotypes

A comparison of clinical characteristics between hMPV genotypes was made for 87 of 93 patients with hMPV infection (Table I). The median age of patients was significantly older (3 vs. 1.5, $P < 0.05$) in children infected with genotype A2 hMPV than in children infected with genotype B1 hMPV, and the range in age was greater for those infected with genotypes A2 and B2 than for those with genotype B1. In genotype A2 hMPV-infected children, the nasal discharge was more frequent whereas wheezing was less frequent than in genotype B1 and B2 hMPV-infected children ($P < 0.05$). In the children infected with genotype B1 hMPV, the most frequent diagnosis was bronchitis (35.7%), but none were diagnosed with pneumonia. Laryngitis was diagnosed in 3 (21.4%) of the 14 children with genotype B1 hMPV, but in none of 30 children infected with

TABLE I. Comparison of Clinical Characteristics Between Human Metapneumovirus (hMPV) Genotype

Variable	hMPV genotype		
	A2 (n = 30)	B1 (n = 14)	B2 (n = 43)
Male sex; no. of patients (%)	15 (50.0)	8 (57.1)	18 (41.9)
Median age of patients (range)	3 (7m–11y)	1.5 (0m–4y)*	2 (9m–13y)
Age; no. of patients (%)			
0–11 months	2 (6.7)	1 (7.1)	2 (4.7)
1–2 years	9 (30.0)	9 (64.3)	21 (48.8)
3–4 years	8 (26.7)	4 (28.6)	12 (27.9)
5–6 years	5 (16.7)	0	3 (7.0)
>6 years	6 (20.0)	0	5 (11.6)
Sign and symptoms; no. of patients (%)			
Cough	30 (100)	14 (100)	42 (97.7)
Nasal discharge	18 (60.0)	5 (35.7)	22 (51.2)
Wheezing	1 (3.3)	4 (28.6)**	11 (25.6)**
Fever	27 (90.0)	12 (85.7)	40 (93.0)
Maximum temperature, median, °C	38.5	38.7	38.8
Duration of fever, median, days (range)[a]	4 (2–6)	4 (0–11)	4 (1–7)
Leukocyte count, median, leukocytes/μl[b]	7,300	9,400	7,900
C reactive protein level, median, mg/dl[b]	0.65	0.8	1.0
Treatment; no. of patients (%)			
Intravenous rehydration	6 (20.0)	5 (35.7)	11 (25.6)
Antibiotics	8 (26.7)	5 (35.7)	12 (27.9)
Final diagnosis; no. of patients (%)			
Pneumonia	1 (3.3)	0	1 (2.3)
Bronchiolitis	0	1 (7.1)	3 (7.0)
Bronchitis	9 (30.0)	5 (35.7)	11 (25.6)
Laryngitis	0	3 (21.4)**	3 (7.0)
Upper-respiratory-tract illness	20 (66.7)	5 (35.7)	24 (55.8)
Gastroenteritis	0	0	1 (2.3)

[a]Number of subjects is 16 for genotype A2, 14 for genotype B1, and 28 for genotype B2.
[b]Number of subjects is for 8 for genotype A2, 5 for genotype B1, and 15 for genotype B2.
*$P < 0.05$ in Mann–Whitney U-test.
**$P < 0.05$ in χ^2 test.

TABLE II. Genotype-Specific Serum Antibody Response in Children With Human Metapneumovirus (hMPV) Infections

Patient no.	Age	Date of respiratory specimen collection	Genotype of isolated hMPV strain	Time from respiratory specimen collection to serum collection, days	hMPV neutralization titers		
					A2	B1	B2
1	1y	June 27, 2005	B1	+7	32	128	128
2	2y	May 2, 2005	A2	+22	128	32	32
		August 5, 2005	B2				
3	10m	July 23, 2005	B2	−28	<[a]	<	<
				+77	8	8	16
4	1y	May 11, 2005	A2	0	<	<	<
				+168	8	<	8

[a]<, Less than 8.

genotype A2 hMPV ($P < 0.05$). Three (7.0%) of the 43 children infected with genotype B2 hMPV were also diagnosed with laryngitis, but there was no significant difference in the frequency compared with that of children infected with genotype A2 hMPV.

Genotype-Specific Neutralization Titers in Children Infected With hMPV

We examined the hMPV neutralization titers of six sera obtained from four children for whom hMPV infection was identified between 2004 and 2006 (Table II). A 1-year-old boy (Patient 1 in Table II), who was infected with the B1 genotype, had a titer of 128 against the B1 and B2 genotypes and a titer of 32 against the A2 genotype 7 days later. A 2-year-old girl (Patient 2 in Table II) infected with the A2 genotype had a titer of 128 against the A2 genotype and a titer of 32 against the B1 and B2 genotypes 22 days later, though she was infected with genotype B2 hMPV 92 days after the initial infection. The antibody titers of the sera that had been obtained 77 and 168 days post-infection ranged from 8 to 16.

Seroprevalence of hMPV Genotype

Sera samples from 213 residents in Yamagata aged from 3 months to 82 years collected between April and September 2005 were applied to serological analysis, and the results are shown in Figure 2. The overall seropositive rates against genotype A2, B1, and B2 hMPV by neutralization assay were 71.8%, 70.0%, and 71.4%, respectively. The seropositive rate increased from 2 years of age and reached a maximum (90%) in children aged 5 years. Among the children aged 1–2 years, the prevalence of antibodies to the B2 genotype was slightly higher than that of antibodies to the A2 genotype, and was obviously higher than that of antibodies to the B1 genotype. There were no apparent differences between the prevalence of antibodies to the A2, B1, and B2 genotypes among the children aged 3–10 years. In the over-10-years group, the prevalence of antibodies to the A2 or B2 genotype was higher than that of antibodies to the B1 genotype, and the median neutralization titer to the A2 genotype was two times higher than those to the B1 and B2 genotypes (data not shown).

DISCUSSION

Our genetic analysis of hMPV isolates revealed that multiple hMPV genotypes cocirculated in the local community over three seasons. The circulation pattern of each genotype was, however, different. Outbreaks of genotype B2 strains occurred annually, but genotype B1 was only prevalent in 2005. Genotype A2 strains circulated in both 2005 and 2006. It appears that outbreaks of each hMPV genotype occur independently. No genotype A1 strains were isolated in Yamagata between 2004 and 2006, and this finding agrees with a previous study by Kaida et al. [2006], which reported that only 1 genotype A1 isolate (from 2004) was found

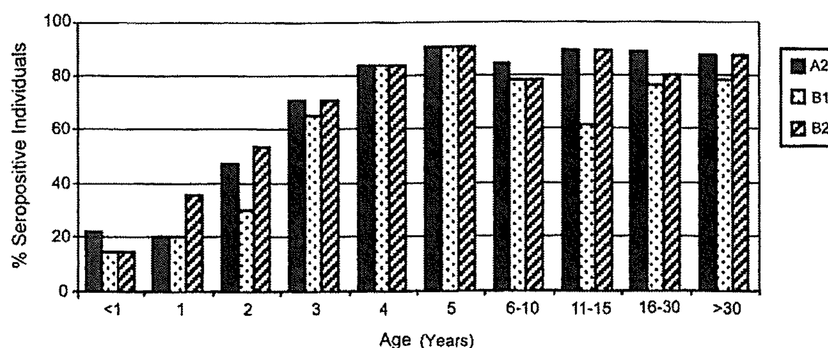

Fig. 2. Prevalence of genotype-specific neutralized antibodies to hMPV detected in 213 residents in Yamagata, categorized by age group. Bars represent the rates of hMPV seropositivity.

among 29 strains isolated in Osaka City, Japan between 2004 and 2005. It appears that few genotype A1 hMPV strains were in circulation in Japan during this period.

A comparison of clinical features by genotype showed that the frequency of laryngitis was higher in children with a genotype B1 hMPV infection whereas wheezing occurred more often in association with genotype B1 and B2 hMPV infections than with genotype A2 hMPV infections ($P < 0.05$). These results suggest that differences in pathogenesis may be dependent on hMPV genotype. The few previous studies that described the relationship between hMPV genotype and disease severity found either no difference [Agapov et al., 2006] or a greater severity of infection associated with genotype A hMPV [Vicente et al., 2006]. We could not compare the severity of illness as all but two children were outpatients: one of those hospitalized was a girl of three who was infected with genotype A2 hMPV and diagnosed with pneumonia, and the other was a girl of two who was infected with genotype B2 hMPV and diagnosed with bronchitis. However, there were no significant differences in maximum temperature, duration of fever, leukocyte count, level of C reactive protein or treatment among children infected with genotype A2, B1, and B2 hMPV. The relationship between virus genotype and severity has been often described for RSV infection. Some studies suggested that infection with subgroup A RSV is associated with more severe illness [Walsh et al., 1997; Gilca et al., 2006], whereas other reports found no differences between RSV subgroups [McIntosh et al., 1993; Martinello et al., 2002]. Gilca et al. [2006] suggested that these discrepancies could be attributed to differences in study design and population, definitions of disease severity, and distribution of genotype shifts from year to year. We agree with this explanation and believe that additional studies on a larger population are needed to define the role of hMPV genotype in pathogenesis.

In this study, the neutralization titer of sera obtained from children with hMPV infection increased against both genotypes A and B, though it was four times higher against the infecting genotype, and then decreased for about 100 days from the time of infection. Although post-infection sera may have cross protection against different genotypes soon after infection, it is unlikely to protect against reinfection with a different genotype from about 100 days after the initial infection; for instance, the 2-year-old girl infected with the A2 genotype was infected with B2 genotype 92 days after the initial infection.

The predominant genotype and the affecting age may be closely related to genotype-specific immune status within a community. In this study, the majority of cases for all genotypes occurred in the younger age groups with lower seropositive rates. There appears to be an association between the lower seropositive rate for genotype B1 among children aged 1–2 years and our result that more than 70% of children infected with the genotype B1 in 2005 were less than 3 years old. The higher seropositive rate for genotype B2 is likely to reflect the outbreak of genotype B2 strains in the previous year in this community. More than 90% of children are infected with hMPV by the age of 5 years; therefore, hMPV infections in children over 5 years of age are probably cases of reinfection. In our study, 30% of children infected with genotype A2 hMPV were over 5 years of age, and were significantly older than children infected with genotype B1 hMPV ($P < 0.05$). Moreover, the median neutralizing antibody titer against the A2 genotype among the residents over 10 years of age was higher than that against B1 and B2 genotypes (data not shown). These finding may suggest that reinfection with A2 genotype was more frequent than that with B1 or B2 genotype. Continued observation within this community is, however, required to determine whether the incidence of reinfection is associated with hMPV genotype.

Finally, we demonstrated that certain clinical characteristics are dependent on hMPV genotype and suggested an association between the hMPV genotype in circulation and genotype-specific seroprevalence, though additional studies are, of course, necessary.

REFERENCES

Abiko C, Mizuta K, Itagaki T, Katsushima N, Ito S, Matsuzaki Y, Okamoto M, Nishimura H, Aoki Y, Murata T, Hoshina H, Hongo S, Ootani K. 2007. Outbreak of human metapneumovirus detected by use of the Vero E6 cell line in isolates collected in Yamagata, Japan, in 2004 and 2005. J Clin Microbiol 45:1912–1919.

Agapov E, Sumino KC, Gaudreault-Keener M, Storch GA, Holtzman MJ. 2006. Genetic variability of human metapneumovirus infection: Evidence of a shift in viral genotype without a change in illness. J Infect Dis 193:396–403.

Bastien N, Ward D, Van Caeseele P, Brandt K, Lee SH, McNabb G, Klisko B, Chan E, Li Y. 2003. Human metapneumovirus infection in the Canadian population. J Clin Microbiol 41:4642–4646.

Boivin G, Abed Y, Pelletier G, Ruel L, Moisan D, Cote S, Peret TC, Erdman DD, Anderson LJ. 2002. Virological features and clinical manifestations associated with human metapneumovirus: A new paramyxovirus responsible for acute respiratory-tract infections in all age groups. J Infect Dis 186:1330–1334.

Ebihara T, Endo R, Kikuta H, Ishiguro N, Yoshioka M, Ma X, Kobayashi K. 2003. Seroprevalence of human metapneumovirus in Japan. J Med Virol 70:281–283.

Gilca R, De Serres G, Tremblay M, Vachon ML, Leblanc E, Bergeron MG, Dery P, Boivin G. 2006. Distribution and clinical impact of human respiratory syncytial virus genotypes in hospitalized children over 2 winter seasons. J Infect Dis 193:54–58.

Kaida A, Iritani N, Kubo H, Shiomi M, Kohdera U, Murakami T. 2006. Seasonal distribution and phylogenetic analysis of human metapneumovirus among children in Osaka City, Japan. J Clin Virol 35: 394–399.

Leung J, Esper F, Weibel C, Kahn JS. 2005. Seroepidemiology of human metapneumovirus (hMPV) on the basis of a novel enzyme-linked immunosorbent assay utilizing hMPV fusion protein expressed in recombinant vesicular stomatitis virus. J Clin Microbiol 43:1213–1219.

Mackay IM, Bialasiewicz S, Jacob KC, McQueen E, Arden KE, Nissen MD, Sloots TP. 2006. Genetic diversity of human metapneumovirus over 4 consecutive years in Australia. J Infect Dis 193:1630–1633.

Martinello RA, Chen MD, Weibel C, Kahn JS. 2002. Correlation between respiratory syncytial virus genotype and severity of illness. J Infect Dis 186:839–842.

Matsuzaki Y, Katsushima N, Nagai Y, Shoji M, Itagaki T, Sakamoto M, Kitaoka S, Mizuta K, Nishimura H. 2006. Clinical features of influenza C virus infection in children. J Infect Dis 193:1229–1235.

McIntosh ED, De Silva LM, Oates RK. 1993. Clinical severity of respiratory syncytial virus group A and B infection in Sydney, Australia. Pediatr Infect Dis J 12:815–819.

Mizuta K, Abiko C, Murata T, Matsuzaki Y, Itagaki T, Sanjoh K, Sakamoto M, Hongo S, Murayama S, Hayasaka K. 2005. Frequent importation of enterovirus 71 from surrounding countries into the local community of Yamagata, Japan, between 1998 and2003. J Clin Microbiol 43:6171–6175.

Peret TC, Boivin G, Li Y, Couillard M, Humphrey C, Osterhaus AD, Erdman DD, Anderson LJ. 2002. Characterization of human metapneumoviruses isolated from patients in North America. J Infect Dis 185:1660–1663.

van den Hoogen BG, de Jong JC, Groen J, Kuiken T, Groot R, Fouchier RA, Osterhaus AD. 2001. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. Nat Med 7:719–724.

van den Hoogen BG, Herfst S, Sprong L, Cane PA, Forleo-Neto E, de Swart RL, Osterhaus AD, Fouchier RA. 2004. Antigenic and genetic variability of human metapneumoviruses. Emerg Infect Dis 10: 658–666.

Vicente D, Montes M, Cilla G, Perez-Yarza EG, Perez-Trallero E. 2006. Differences in clinical severity between genotype A and genotype B human metapneumovirus infection in children. Clin Infect Dis 42:e111–e113.

Walsh EE, McConnochie KM, Long CE, Hall CB. 1997. Severity of respiratory syncytial virus infection is related to virus strain. J Infect Dis 175:814–820.

Williams JV, Harris PA, Tollefson SJ, Halburnt-Rush LL, Pingsterhaus JM, Edwards KM, Wright PF, Crowe JE. 2004. Human metapneumovirus and lower respiratory tract disease in otherwise healthy infants and children. N Engl J Med 350:443–450.

Williams JV, Wang CK, Yang CF, Tollefson SJ, House FS, Heck JM, Chu M, Brown JB, Lintao LD, Quinto JD, Chu D, Spaete RR, Edwards KM, Wright PF, Crowe JE. 2006. The role of human metapneumovirus in upper respiratory tract infections in children: A 20-year experience. J Infect Dis 193:387–395.

Immunology Letters 118 (2008) 88–95

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/

Interferon-γ enhances human eosinophil effector functions induced by granulocyte-macrophage colony-stimulating factor or interleukin-5

Takafumi Yamaguchi [a,c], Hirokazu Kimura [b], Masahiko Kurabayashi [c], Kunihisa Kozawa [a], Masahiko Kato [a,d,*]

[a] Gunma Prefectural Institute of Public Health and Environmental Sciences, 378, Kamioki-machi, Maebashi, Gunma 371-0052, Japan
[b] Department of Infectious Diseases Surveillance Center, National Institute of Infectious Diseases, 4-7-1, Gakuen, Musashimurayama, Tokyo 208-0011, Japan
[c] Department of Medicine and Biological Science, Gunma University Graduate School of Medicine, 3-39-22, Showa-machi, Maebashi, Gunma 371-8511, Japan
[d] Department of Allergy and Immunology, Gunma Children's Medical Center, 779, Hokkitsu-machi, Shibukawa, Gunma 377-8577, Japan

ARTICLE INFO

Article history:
Received 28 January 2008
Received in revised form 12 March 2008
Accepted 17 March 2008
Available online 10 April 2008

Keywords:
Interferon-γ
Eosinophils
Granulocyte-macrophage colony-stimulating factor
Interleukin-5
Superoxide anion generation

ABSTRACT

T helper (Th) 2-type cytokines play a dominant role in allergic inflammation. Accumulating evidence suggests that Th1-type cytokines antagonize Th2-type cytokine responses; however, recent studies demonstrate that Th1 cytokines might enhance Th2 immune responses. We examined whether interferon (IFN)-γ, a representative Th1 cytokine, modifies the effector functions of human eosinophils stimulated by granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-5. GM-CSF and IL-5 have significant functional homology, and contribute to the regulation of Th2 immunity. After the pretreatment of eosinophils with IFN-γ, GM-CSF- or IL-5-induced eosinophil functions were examined, including superoxide anion generation, degranulation, adhesion, expression of GM-CSF receptor (R), IL-5R, or CD11b, and phosphorylation of intracellular signaling molecules. Superoxide anion generation was measured using the cytochrome *c* reduction method. Degranulation and cell adhesion were evaluated based on eosinophil-derived neurotoxin (EDN) contents in supernatants or adherent cells. Phosphorylation of signaling molecules was analyzed using a multiplex beads array system. Preincubation with IFN-γ resulted in enhanced GM-CSF- or IL-5-induced superoxide anion generation and degranulation of human eosinophils, whereas stimulus-induced eosinophil adhesion was unaffected. In addition, IFN-γ did not influence the expression of GM-CSFR, IL-5R, and CD11b. Furthermore, IFN-γ upregulated GM-CSF- or IL-5-induced phosphorylation of extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), and activating transcription factor (ATF)-2. Finally, we confirmed that MAPK inhibitors blocked the enhancement of stimuli-induced superoxide anion generation of IFN-γ treated eosinophils. In conclusion, IFN-γ might upregulate ERK, p38, or JNK/ATF-2 phosphorylation induced by GM-CSF or IL-5, leading to enhanced cytokine-induced eosinophil superoxide generation and degranulation.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Eosinophils are involved in the pathogenesis of allergic diseases, such as bronchial asthma, allergic rhinitis, and atopic dermatitis [1]. These allergic diseases are closely related to T helper (Th) 2 immune responses, which are characterized by high levels of cytokines including interleukin (IL)-4, IL-5, IL-9, and IL-13 [2]. These cytokines orchestrate the recruitment and activation of different effector cells, such as eosinophils [3]. It has been recognized that altering the cytokine-producing profile of Th2 cells by inducing Th1 responses may be protective against Th2-related diseases [4].

Interferon (IFN)-γ is a representative Th1 effector cytokine and is closely involved in protecting cells from viral infections [5]. Furthermore, it has been shown to play a crucial role in inhibiting Th2 responses and Th2 immunity. Th1-stimulating activity of lung macrophages inhibits Th2-mediated allergic airway inflammation by an IFN-γ-dependent mechanism [6]. IFN-γ exerts direct inhibitory effects on Th2 cytokines, reducing the levels of IL-4 and IL-5 production. The IFN-γ signaling pathway activates T-bet protein, the Th1-specific and Th2-suppressing transcription factor. In fact, ectopic expression of T-bet represses IL-4 and IL-5 in Th2

Abbreviations: ATF-2, Activating transcription factor-2; DMSO, dimethyl sulfoxide; EDN, eosinophil-derived neurotoxin; ERK, extracellular signal-regulated kinase; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NADPH, nicotinamide adenine dinucleotide phosphate; PMN, polymorphonuclear leukocytes; RSV, respiratory syncytial virus; Th, T helper.

* Corresponding author. Tel.: +81 27 232 4881; fax: +81 27 234 8438.
E-mail address: mkato@gcmc.pref.gunma.jp (M. Kato).

0165-2478/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.imlet.2008.03.005

cells [7]. IFN-γ also has a crucial role in inhibiting Th2 responses, but not only through T-bet expression. Loss of IL-4 receptor responsiveness may be another mechanism that suppresses Th2 development in polarizing Th1 cells [8]. Another critical role of IFN-γ in allergic reactions is its ability to inhibit immunoglobulin class switching to IgE, an important mediator of allergic pathological states induced by Th2 cytokines [9]. Additionally, *in vivo* models of allergic inflammation have also demonstrated the regulation of allergen-induced eosinophilic infiltration by IFN-γ. Recombinant IFN-γ treatment before inhalation of aerosolized antigen prevents eosinophil infiltration into the trachea of sensitized mice [10]. It has also been demonstrated that IFN-γ is responsible for regulating the activation, differentiation and recruitment of eosinophils [11,12]. Furthermore, Th2 cytokine production is enhanced in IFN-γ knockout mice [13].

Despite these reports, the role of IFN-γ in promoting Th1- and inhibiting Th2-type responses has also been the subject of some controversy. Bocek et al. demonstrated that exogenous IFN-γ enhanced *in vivo* Th2 priming in an IFN-γ-deficient mouse [14]. The proportions of IL-4-producing $CD4^{+}$ cells were lower in IFN-γ −/− mice than in wild-type mice. Administration of IFN-γ caused an increase in the proportion of T cells secreting IL-4. These results suggest that efficient *in vivo* Th2 priming requires a small amount of IFN-γ. Juntti et al. have shown that children with asthma hospitalized for respiratory syncytial virus (RSV) infection in infancy have significantly higher concentrations of IFN-γ than controls with asthma without RSV infection [15], suggesting that an increase in IFN-γ after an early RSV infection may be related to the pathogenesis of asthma. In addition, IFN-γ promoted eosinophil survival rather than apoptosis *in vitro* [16]. Furthermore, Fujii et al. demonstrated that expression of cysteinyl leukotriene type-2 receptors (CysLT2R) on eosinophils increases in patients during asthma exacerbation, especially in nonatopic subjects, and is upregulated by IFN-γ. The authors therefore speculate that a pathway via CysLT2R might modulate exacerbation of asthma [17].

In this study, we examined whether IFN-γ influenced effector functions, such as superoxide anion generation and degranulation, in GM-CSF- or IL-5-induced human eosinophils. GM-CSF has a common β receptor subunit to IL-5, which is a representative Th2 cytokine. These cytokines have significant functional homology, and contribute to the regulation of Th2 immunity [18]. The aim of the present study is to elucidate IFN-γ implicated in the function of GM-CSF- or IL-5-activated eosinophils. This experimental approach provides clarification of the association between Th1 cytokines and allergic asthma.

2. Materials and methods

2.1. Reagents

IFN-γ, GM-CSF, and IL-5 were purchased from R&D Systems (Minneapolis, MN). IFN-γ was dissolved in PBS, 0.1% human serum albumin (HSA) at 100 μg/ml (10^6 U/ml) and stored at −80 °C. GM-CSF and IL-5 were dissolved in PBS, 0.1% HSA at 10 μg/ml and stored at −80 °C. HSA and cytochrome *c* were purchased from Sigma (St. Louis, MO). FITC-conjugated anti-GM-CSFR, PE-conjugated anti-IL-5R, PE-conjugated anti-CD11b mouse monoclonal antibody, and mouse IgG1 control immunoglobulin were purchased from Becton Dickinson (San Jose, CA). The Bio-Plex 12-plex Phosphoprotein Assay Kit and the Phosphoprotein Testing Reagent Kit were purchased from Bio-Rad Laboratories (Hercules, CA). Extracellular signal-regulated kinase (ERK) inhibitor peptide II, SB 202190 and c-Jun N-terminal kinase (JNK) inhibitor I were obtained from Calbiochem (La Jolla, CA). Inhibitors were dissolved in dimethyl sulfoxide (DMSO) and stored at −80 °C.

2.2. Cell preparation

Eosinophils were purified by a previously described method using a magnetic cell separation system (MACS; Becton Dickinson, San Jose, CA) [19,20]. Briefly, heparinized blood was obtained from healthy donors and diluted with an equal volume of PBS. Diluted blood was layered over Histopaque solution (density, 1.083 g/ml; Sigma) and centrifuged at 1000 × *g* for 30 min. The supernatant was then removed and erythrocyte pellets were subjected to two cycles of hypotonic water lysis. Isolated granulocytes were washed with PIPES (Sigma) buffer containing 1% FBS (JRH Biosciences, Lenexa, KS), and an equal volume of anti-CD16 antibody-conjugated magnetic particles (Miltenyi Biotec GmbH, Bergish Gladbach, Germany) was added to the cell pellet. After 45 min on ice, cells were resuspended and loaded onto a separation column positioned in a strong magnetic field for MACS. Cells were eluted three times. Purity of eosinophils was determined by counting Randolph's stain in more than 98% of them. Cell viability always exceeded 98%, as determined by trypan blue exclusion and propidium iodide staining. Purified eosinophils (1×10^6 cells/ml) were suspended in RPMI 1640 with 25 mM HEPES, 10% FBS and treated with or without IFN-γ (500 U/ml) for 4 h at 37 °C. In preliminary studies, there was no difference in cell viability between IFN +/− cells (data not shown).

2.3. Superoxide anion generation

Superoxide anion generation was measured using a cytochrome *c* reduction method, as previously described [21,22]. After preincubation with or without IFN-γ, eosinophils were suspended in HBSS with 10 mM HEPES, 0.01% gelatin at 5×10^5 cells/ml. Fifty microliters of GM-CSF, IL-5 or medium were dispensed into HSA-coated, flat-bottom 96-well tissue culture plates, followed by 100 μl of cell suspension. Immediately following the addition of 100 μM cytochrome *c*, reaction wells were measured for optical density at 550 nm in a microplate autoreader (SPECTRA MAX 190 microplate reader, Molecular Devices) for 3 h. Superoxide anion generation was calculated using $\varepsilon_{550} = 21.1 \times 10^3\ \text{cm}^{-1}\,\text{M}^{-1}$ for reduced cytochrome and expressed as nM of superoxide anion generation/10^5 cells. In experiments using mitogen-activated protein kinase (MAPK) inhibitors, eosinophils treated with or without IFN-γ were suspended in HBSS with 10 mM HEPES, 0.01% gelatin at 10^6 cells/ml. Fifty microliters of the cells were preincubated with 50 μl of medium, ERK inhibitor peptide II, SB 202190, or JNK inhibitor I for 30 min at 37 °C in HSA-coated, flat-bottom 96-well tissue culture plates. After incubation, the cells were stimulated with 1 ng/ml of GM-CSF or IL-5. Immediately following the addition of 100 μM cytochrome *c*, the reaction wells were measured for optical density at 550 nm in a microplate autoreader for up to 3 h.

2.4. Eosinophil degranulation

Eosinophil degranulation was assessed as previously described [23,24]. Briefly, after preincubation with or without IFN-γ, eosinophils were suspended in RPMI 1640 with 25 mM HEPES, 0.01% gelatin at 10^6 cells/ml. One hundred microliters of cells were dispensed in HSA-coated, flat-bottom 96 well tissue culture plates followed by 100 μl of GM-CSF, IL-5, or medium. After 3 h of incubation, the supernatants were collected and stored at −20 °C until enzyme immunoassay (EDN ELISA Kit, MBL) for eosinophil-derived neurotoxin (EDN) content to quantitate eosinophil degranulation. Total cellular EDN content was measured simultaneously in supernatants from cells lysed with 0.5% Nonidet P-40 detergent. All experiments were performed in duplicate.