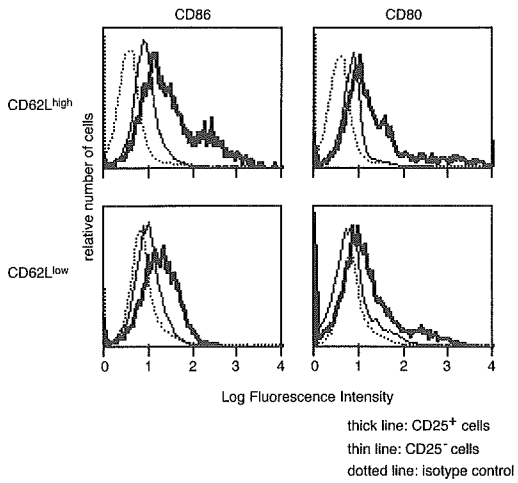

FIGURE 7. T cells isolated from 12-day tumor-draining LNs were analyzed for the expression of CD80 or CD86. CD4$^+$ T cells positively selected with magnetic beads were three-color-stained with FITC-labeled anti-CD80 mAb or FITC-labeled anti-CD86 mAb in the presence of PE-labeled anti-CD62L mAb and PerCP-labeled anti-CD25 mAb. Gated CD62L^{high}CD4$^+$CD25$^+$, CD62L^{high}CD4$^+$CD25$^-$,CD62L^{low}CD4$^+$CD25$^+$, or CD62L^{low}CD4$^+$CD25$^-$ T cells were analyzed for CD80 or CD86 expression. A total of 10^7 cells were analyzed for each sample.

CD62L^{high}CD4$^+$CD25$^+$ regulatory T cells suppress effector CD4$^+$ T cell functions via CD86/CTLA-4 T-T interactions

It has been reported that an inhibitory Ab against CTLA-4 could abrogate the induction of suppression by CD4$^+$CD25$^+$ Treg, and CTLA-4 expressed on Treg was thought to be important for this phenomenon (27). However, the mechanism by which Treg deliver a regulatory signal to effector T cells is still unclear. We examined the expression of CD80 and CD86 on fractionated tumor-draining LN T cells. Unexpectedly, only the CD62L^{high}CD4$^+$CD25$^+$ T cells had a subpopulation that expressed CD86 (Fig. 7). CD62L^{low}CD4$^+$ or CD62L^{high}CD4$^+$CD25$^-$ T cells did not express a detectable level of CD86 even after stimulation with CD3 (data not shown). Next, we tested whether CD86 on regulatory T cells participates in the suppression of T cell functions. CD62L^{low} T cells and CD62L^{high}CD4$^+$CD25$^+$ Treg cells were stimulated with immobilized anti-CD3 mAb in the presence or the absence of an inhibitory mAb against CD80, CD86, or CTLA-4. Anti-CTLA-4 as well as anti-CD86 inhibitory mAb prevented the suppression of CD62L^{low}CD4$^+$ T cells induced by CD62L^{high}CD4$^+$CD25$^+$ Treg cells, whereas anti-CD80 inhibitory mAb or isotype-matched irrelevant Abs did not have any influence (Fig. 8*a*). However, blockade of CTLA-4 or CD86 could not reverse the suppression of CD62L^{low}CD8$^+$ LN T cells (Fig. 8*b*).

Discussion

In this study we demonstrated that both effector T cells and Treg cells are primed in the same LNs that drain growing MCA 205 s.c. tumors. The antitumor effector T cells in tumor-draining LNs belong to a subpopulation that down-regulated CD62L expression, as we previously demonstrated (11, 14). In contrast, the CD62L^{high}CD4$^+$CD25$^+$ subpopulation in tumor-draining LNs abrogate the in vivo antitumor therapeutic efficacy of CD62L^{low} antitumor LN T cells (Fig. 1). Kinetic analyses indicate that CD62L^{high}CD4$^+$CD25$^+$ Treg proliferated in tumor-draining LNs during tumor progression (Fig. 6). Data obtained in vitro reveal that the Treg generated in tumor-draining LNs to abrogate antitumor reactivity possess the same functional properties and level of *foxp3* expression as the Treg that naturally arise in the thymus to maintain self-tolerance (Figs. 2–5).

CD62L^{high}CD4$^+$CD25$^+$ T cells derived from spleens of naive mice could not abrogate antitumor reactivity in vivo (Fig. 1). Moreover, Treg cells from naive mice did not inhibit the production of IFN-γ by antitumor effector T cells upon Ag stimulation by DCs acquired from apoptotic tumor cells (Fig. 2*b*), even though they had comparable suppressive activity as the Treg cells generated in tumor-draining LNs upon nonspecific CD3 stimulation.

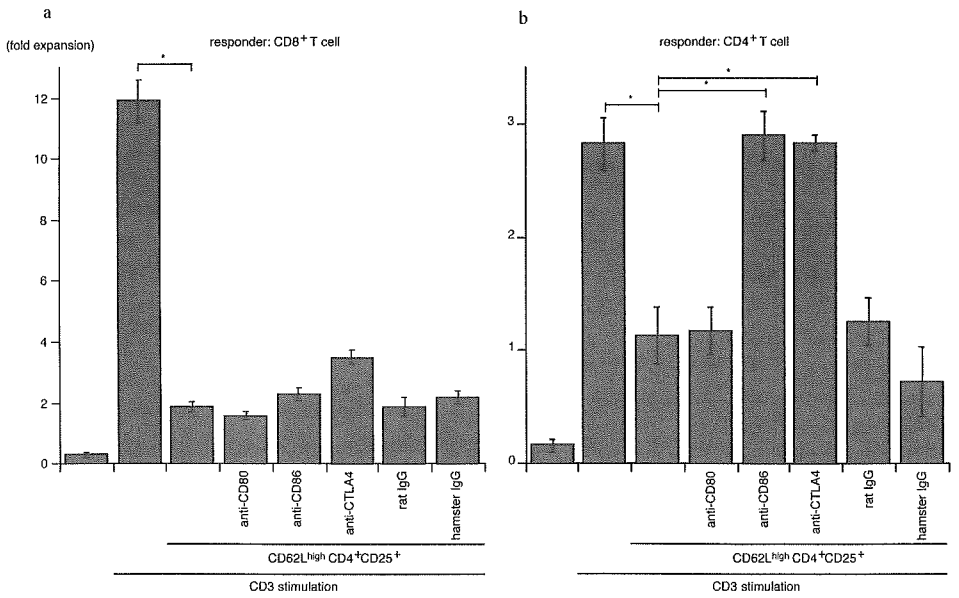

FIGURE 8. Proliferation assay of T cells in the presence or the absence of a blockade between CD86 and CTLA-4. CD62L^{low}CD4$^+$ or CD8$^+$ T cells (1×10^5) were cocultured with 5×10^4 CD62L^{high}CD4$^+$CD25$^+$ T cells in 200 μl of CM on 96-well plates with immobilized anti-CD3 mAb for 48 h. T cells were isolated from LNs draining MCA 205 s.c. tumors for 12 days. To inhibit the interaction between CD86 and CTLA-4, anti-CD86 mAb or anti-CTLA-4 mAb was added to the medium at 10 μg/ml during the 48 h of stimulation. T cells were recultured in 1 ml of CM supplemented with 10 U/ml IL-2 on 24-well plates at 1×10^5/ml. As a control, anti-CD80 mAb, rat IgG, or hamster IgG was added at the same concentration. Three wells were examined for each condition, and cells were enumerated every 24 h. The *y*-axis indicates the fold increase in number on day 2. *, $p < 0.01$.

Hence, it is likely that Treg are required to receive TCR/CD3 signaling to interfere with the function of effector T cells and that the Treg generated in tumor-draining LNs recognize tumor-associated Ag.

Although it has been demonstrated that $CD62L^{high}CD4^+CD25^+$ T cells possess superior regulatory activity in several systems, $CD62L^{low}CD4^+CD25^+$ T cells were still considered Treg cells (15, 16, 18). However, the differences in *foxp3* expression and priming kinetics suggest that the $CD62L^{low}CD4^+CD25^+$ T cell subpopulation in tumor-draining LNs is distinct from $CD62L^{high}CD4^+CD25^+$ T cells (Figs. 5*A* and 6). $CD62L^{low}CD4^+CD25^+$ T cells produced even more IFN-γ than $CD62L^{low}CD4^+CD25^-$ T cells upon CD3 stimulation (Fig. 4). Furthermore, $CD62L^{low}CD4^+CD25^+$ LN T cells mediated antitumor efficacy in vivo (Fig. 1). These results indicated that $CD62L^{low}CD4^+CD25^+$ T cells are effector T cells that express CD25 because of recent TCR stimulation before clonal expansion in LNs.

Little is known about the trafficking of Treg cells; however, our study shows that Treg cells generated in tumor-draining LNs have an up-regulated expression of CD62L, VLA-4, and LFA-1 (Figs. 1 and 5*B*). CD62L recognizes specific ligands on high endothelial venules and is considered the homing receptor for secondary lymphoid tissues (30). In contrast, VLA-4 and LFA-1 are thought to play a central role in T cell trafficking to inflammatory sites by recognizing VCAM-1 and ICAM-1 on endothelial cells. Thus, it is likely that $CD62L^{high}CD4^+CD25^+$ Treg cells can follow either $CD62L^{high}$ naive T cells to suppress priming in secondary lymphoid organs or activated $CD62L^{low}$ effector T cells that express VLA-4 and LFA-1 to suppress immune reactions in the effector phase.

$CD62L^{high}CD4^+CD25^+$ Treg cells suppressed either $CD4^+$ or $CD8^+$ effector T cell functions, including cytokine production and cell proliferation, in the absence of APC upon stimulation with CD3 in a cell-cell contact-dependent manner (Fig. 2. *c* and *d*, and Fig. 3). Hence, the regulatory signals sent to either $CD4^+$ or $CD8^+$ effector T cells were received directly from $CD62L^{high}CD4^+CD25^+$ Treg cells and were not mediated by APC. It is postulated that ligation of CD80 and/or CD86 expressed on effector T cells by CTLA-4 on Treg cells causes outside-in signaling and results in suppression (31), although it is well documented that $p56^{lck}$-induced tyrosine phosphorylation, which is the major signal pathway of the TCR/CD3 complex, can be reversed by CTLA-4 ligation (32). We found that the $CD62L^{high}CD4^+CD25^+$ Treg cells contained a subpopulation that expressed CD86 in tumor-draining LNs (Fig. 7). Effector $CD62L^{low}CD4^+$ or naive $CD62L^{high}CD4^+CD25^-$ T cells derived from tumor-draining LNs did not express CD86 or CD80 even after 48-h stimulation with immobilized anti-CD3 mAb (data not shown). The inhibitory mAb against CTLA-4 or CD86, but not CD80, completely reversed the inhibitory effect on the proliferation of $CD4^+$ effector LN T cells by $CD62L^{high}CD4^+CD25^+$ T cells (Fig. 8). In contrast, the same treatment could not reverse the suppression of $CD8^+$ effector LN T cells. $CD62L^{low}CD4^+$ T cells, but not $CD8^+$ T cells, isolated from tumor-draining LNs expressed CTLA-4 (Fig. 5*B*). Our data suggested that CD86 exclusively expressed on Treg cells plays a pivotal role in regulating $CD4^+$ effector T cells by interacting with CTLA-4 on effector T cells. This is a novel mechanism by which Treg cells suppress effector T cell function. This finding is compatible with recent reports demonstrating that $CD4^+CD25^+$ T cells derived from CTLA-4-deficient mice mediated suppression (33) and that T cells transfected with cDNA encoding CD86 suppressed graft-vs-host disease (34). The reason why the bioactivity of CD86 on T cells is different from that on APC is unclear; however, DCs are capable of selecting the receptor of B7 by recruiting either CD28 or CTLA-4 into immunological synapses (35). In contrast, it is unlikely that Treg cells have the ability to develop immunological synapse with effector T cells to select receptors. Moreover, it was demonstrated that CD86 expressed on T cells had a hypoglycosylated form and showed no detectable binding activity to CD28 with preserved binding to CTLA-4 (36). Thus, it is possible that CD86 on T cells preferentially give negative signaling through CTLA-4 (37).

$CD62L^{high}CD4^+CD25^+$ T cells regulated $CD8^+$ T cell functions via mechanisms other than the CD86-CTLA-4 interaction, because the inhibitory mAb against CD86 or CTLA-4 did not abolish the suppression. Because neutralizing Ab against TGF-β partially inhibited the induction of suppression, it is likely that membrane-bound TGF-β participated in $CD8^+$ T cell suppression (data not shown).

These results indicate that Treg cells for tumor Ags are primed in draining LNs during tumor progression and that the balance between $CD62L^{low}$ effector T cell priming and $CD62L^{high}CD4^+CD25^+$ Treg cell priming in secondary lymphoid organs determines the outcome of antitumor immune reactions. Our data also indicate that promoting antitumor effector $CD62L^{low}$ T cell priming while eliminating $CD62L^{high}CD4^+CD25^+$ Treg cells or inhibiting regulatory mechanisms such as CD86-CTLA-4 interaction is critical to establishing effective antitumor immunotherapy. Furthermore, it might be possible to orchestrate adaptive immune reactions by manipulating the balance of effector and regulatory T cell priming against acquired Ags, such as infectious pathogens, alloantigens, and allergens.

Disclosures

The authors have no financial conflict of interest.

References

1. Frazer, I. H., R. De Kluyver, G. R. Leggatt, H. Y. Guo, L. Dunn, O. White, C. Harris, A. Liem, and P. Lambert. 2001. Tolerance or immunity to a tumor antigen expressed in somatic cells can be determined by systemic proinflammatory signals at the time of first antigen exposure. *J. Immunol.* 167: 6180–6187.
2. Shimizu, J., S. Yamazaki, and S. Sakaguchi. 1999. Induction of tumor immunity by removing $CD25^+CD4^+$ T cells: a common basis between tumor immunity and autoimmunity. *J. Immunol.* 163: 5211–5218.
3. Sakaguchi, S., N. Sakaguchi, J. Shimizu, S. Yamazaki, T. Sakihama, M. Itoh, Y. Kuniyasu, T. Nomura, M. Toda, and T. Takahashi. 2001. Immunologic tolerance maintained by $CD25^+$ $CD4^+$ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol. Rev.* 182: 18–32.
4. Meloni, F., P. Vitulo, A. M. Bianco, E. Paschetto, M. Morosini, A. Cascina, I. Mazzucchelli, L. Ciardelli, T. Oggionni, A. M. Fietta, et al. 2004. Regulatory $CD4^+CD25^+$ T cells in the peripheral blood of lung transplant recipients: correlation with transplant outcome. *Transplantation* 77: 762–766.
5. Suvas, S., U. Kumaraguru, C. D. Pack, S. Lee, and B. T. Rouse. 2003. $CD4^+CD25^+$ T cells regulate virus-specific primary and memory $CD8^+$ T cell responses. *J. Exp. Med.* 198: 889–901.
6. Green, E. A., L. Gorelik, C. M. McGregor, E. H. Tran, and R. A. Flavell. 2003. $CD4^+CD25^+$ T regulatory cells control anti-islet $CD8^+$ T cells through TGF-β-TGF-β receptor interactions in type 1 diabetes. *Proc. Natl. Acad. Sci. USA* 100: 10878–10883.
7. Tanaka, H., J. Tanaka, J. Kjaergaard, and S. Shu. 2002. Depletion of $CD4^+$ $CD25^+$ regulatory cells augments the generation of specific immune T cells in tumor-draining lymph nodes. *J. Immunother.* 25: 207–217.
8. Takahashi, T., and S. Sakaguchi. 2003. Naturally arising $CD25^+CD4^+$ regulatory T cells in maintaining immunologic self-tolerance and preventing autoimmune disease. *Curr. Mol. Med.* 3: 693–706.
9. Walker, M. R., D. J. Kasprowicz, V. H. Gersuk, A. Benard, M. Van Landeghen, J. H. Buckner, and S. F. Ziegler. 2003. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human $CD4^+CD25^-$ T cells. *J. Clin. Invest.* 112: 1437–1443.
10. Walker, L. S., A. Chodos, M. Eggena, H. Dooms, and A. K. Abbas. 2003. Antigen-dependent proliferation of $CD4^+$ $CD25^+$ regulatory T cells in vivo. *J. Exp. Med.* 198: 249–258.
11. Kagamu, H., J. E. Touhalisky, G. E. Plautz, J. C. Krauss, and S. Shu. 1996. Isolation based on L-selectin expression of immune effector T cells derived from tumor-draining lymph nodes. *Cancer Res.* 56: 4338–4342.
12. Watanabe, S., H. Kagamu, H. Yoshizawa, N. Fujita, H. Tanaka, J. Tanaka, and F. Gejyo. 2003. The duration of signaling through CD40 directs biological ability of dendritic cells to induce antitumor immunity. *J. Immunol.* 171: 5828–5836.

13. Fujita, N., H. Kagamu, H. Yoshizawa, K. Itoh, H. Kuriyama, N. Matsumoto, T. Ishiguro, J. Tanaka, E. Suzuki, H. Hamada, et al. 2001. CD40 ligand promotes priming of fully potent antitumor CD4$^+$ T cells in draining lymph nodes in the presence of apoptotic tumor cells. *J. Immunol.* 167: 5678–5688.
14. Kagamu, H., and S. Shu. 1998. Purification of L-selectin^{low} cells promotes the generation of highly potent CD4 antitumor effector T lymphocytes. *J. Immunol.* 160: 3444–3452.
15. Szanya, V., J. Ermann, C. Taylor, C. Holness, and C. G. Fathman. 2002. The subpopulation of CD4$^+$CD25$^+$ splenocytes that delays adoptive transfer of diabetes expresses L-selectin and high levels of CCR7. *J. Immunol.* 169: 2461–2465.
16. Fu, S., A. C. Yopp, X. Mao, D. Chen, N. Zhang, M. Mao, Y. Ding, and J. S. Bromberg. 2004. CD4$^+$ CD25$^+$ CD62$^+$ T-regulatory cell subset has optimal suppressive and proliferative potential. *Am. J. Transplant* 4: 65–78.
17. Godfrey, W. R., Y. G. Ge, D. J. Spoden, B. L. Levine, C. H. June, B. R. Blazar, and S. B. Porter. 2004. In vitro-expanded human CD4$^+$CD25$^+$ T-regulatory cells can markedly inhibit allogeneic dendritic cell-stimulated MLR cultures. *Blood* 104: 453–461.
18. Taylor, P. A., A. Panoskaltsis-Mortari, J. M. Swedin, P. J. Lucas, R. E. Gress, B. L. Levine, C. H. June, J. S. Serody, and B. R. Blazar. 2004. L-selectin^{hi} but not the L-selectin^{lo} CD4$^+$25^{++} T-regulatory cells are potent inhibitors of GVHD and BM graft rejection. *Blood* 104: 3804–3812.
19. Shu, S. Y., and S. A. Rosenberg. 1985. Adoptive immunotherapy of newly induced murine sarcomas. *Cancer Res.* 45: 1657–1662.
20. Yoshizawa, H., A. E. Chang, and S. Shu. 1991. Specific adoptive immunotherapy mediated by tumor-draining lymph node cells sequentially activated with anti-CD3 and IL-2. *J. Immunol.* 147: 729–737.
21. Kuniyasu, Y., T. Takahashi, M. Itoh, J. Shimizu, G. Toda, and S. Sakaguchi. 2000. Naturally anergic and suppressive CD25$^+$CD4$^+$ T cells as a functionally and phenotypically distinct immunoregulatory T cell subpopulation. *Int. Immunol.* 12: 1145–1155.
22. Tommasini, A., S. Ferrari, D. Moratto, R. Badolato, M. Boniotto, D. Pirulli, L. D. Notarangelo, and M. Andolina. 2002. X-chromosome inactivation analysis in a female carrier of FOXP3 mutation. *Clin. Exp. Immunol.* 130: 127–130.
23. Schubert, L. A., E. Jeffery, Y. Zhang, F. Ramsdell, and S. F. Ziegler. 2001. Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation. *J. Biol. Chem.* 276: 37672–37679.
24. Khattri, R., T. Cox, S. A. Yasayko, and F. Ramsdell. 2003. An essential role for scurfin in CD4$^+$CD25$^+$ T regulatory cells. *Nat. Immunol.* 4: 337–342.
25. Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. Foxp3 programs the development and function of CD4$^+$CD25$^+$ regulatory T cells. *Nat. Immunol.* 4: 330–336.
26. Shimizu, J., S. Yamazaki, T. Takahashi, Y. Ishida, and S. Sakaguchi. 2002. Stimulation of CD25$^+$CD4$^+$ regulatory T cells through GITR breaks immunological self-tolerance. *Nat. Immunol.* 3: 135–142.
27. Takahashi, T., T. Tagami, S. Yamazaki, T. Uede, J. Shimizu, N. Sakaguchi, T. W. Mak, and S. Sakaguchi. 2000. Immunologic self-tolerance maintained by CD25$^+$CD4$^+$ regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J. Exp. Med.* 192: 303–310.
28. McHugh, R. S., M. J. Whitters, C. A. Piccirillo, D. A. Young, E. M. Shevach, M. Collins, and M. C. Byrne. 2002. CD4$^+$CD25$^+$ immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity* 16: 311–323.
29. Huang, C. T., C. J. Workman, D. Flies, X. Pan, A. L. Marson, G. Zhou, E. L. Hipkiss, S. Ravi, J. Kowalski, H. I. Levitsky, et al. 2004. Role of LAG-3 in regulatory T cells. *Immunity* 21: 503–513.
30. Yeh, J. C., N. Hiraoka, B. Petryniak, J. Nakayama, L. G. Ellies, D. Rabuka, O. Hindsgaul, J. D. Marth, J. B. Lowe, and M. Fukuda. 2001. Novel sulfated lymphocyte homing receptors and their control by a Core1 extension β1,3-*N*-acetylglucosaminyltransferase. *Cell* 105: 957–969.
31. Paust, S., L. Lu, N. McCarty, and H. Cantor. 2004. Engagement of B7 on effector T cells by regulatory T cells prevents autoimmune disease. *Proc. Natl. Acad. Sci. USA* 101: 10398–10403.
32. Lee, K. M., E. Chuang, M. Griffin, R. Khattri, D. K. Hong, W. Zhang, D. Straus, L. E. Samelson, C. B. Thompson, and J. A. Bluestone. 1998. Molecular basis of T cell inactivation by CTLA-4. *Science* 282: 2263–2266.
33. Tang, Q., E. K. Boden, K. J. Henriksen, H. Bour-Jordan, M. Bi, and J. A. Bluestone. 2004. Distinct roles of CTLA-4 and TGF-β in CD4$^+$CD25$^+$ regulatory T cell function. *Eur. J. Immunol.* 34: 2996–3005.
34. Taylor, P. A., C. J. Lees, S. Fournier, J. P. Allison, A. H. Sharpe, and B. R. Blazar. 2004. B7 expression on T cells down-regulates immune responses through CTLA-4 ligation via T-T interactions. *J. Immunol.* 172: 34–39.
35. Pentcheva-Hoang, T., J. G. Egen, K. Wojnoonski, and J. P. Allison. 2004. B7-1 and B7-2 selectively recruit CTLA-4 and CD28 to the immunological synapse. *Immunity* 21: 401–413.
36. Hollsberg, P., C. Scholz, D. E. Anderson, E. A. Greenfield, V. K. Kuchroo, G. J. Freeman, and D. A. Hafler. 1997. Expression of a hypoglycosylated form of CD86 (B7-2) on human T cells with altered binding properties to CD28 and CTLA-4. *J. Immunol.* 159: 4799–4805.
37. Greenfield, E. A., E. Howard, T. Paradis, K. Nguyen, F. Benazzo, P. McLean, P. Hollsberg, G. Davis, D. A. Hafler, A. H. Sharpe, et al. 1997. B7.2 expressed by T cells does not induce CD28-mediated costimulatory activity but retains CTLA4 binding: implications for induction of antitumor immunity to T cell tumors. *J. Immunol.* 158: 2025–2034.

Kidney International, Vol. 68 (2005), pp. 188–192

CLINICAL NEPHROLOGY – EPIDEMIOLOGY – CLINICAL TRIALS

A novel type of encephalopathy associated with mushroom Sugihiratake ingestion in patients with chronic kidney diseases

FUMITAKE GEJYO, NORIYUKI HOMMA, NOBORU HIGUCHI, KEN ATAKA, TOMOKO TERAMURA, BASSAM ALCHI, YUKIO SUZUKI, SCHINICHI NISHI, ICHIEI NARITA, and THE JAPANESE SOCIETY OF NEPHROLOGY

Division of Clinical Nephrology and Rheumatology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan; Internal Medicine, Niigata Prefectural Shibata Hospital, Niigata, Japan; Internal Medicine, Tsuruoka Shonai Hospital, Yamagata Prefectural Hospital, Tsuruoka, Japan; Internal Medicine, Hanazono Hospital, Akita Prefectural Hosopital, Akita, Japan; and Niigata Prefectural Bureau of Health and Welfare, Niigata, Japan

A novel type of encephalopathy associated with mushroom Sugihiratake ingestion in patients with chronic kidney diseases.

Background. The etiology of encephalopathy in uremic patients is multiple. We recently encountered a novel type of encephalopathy which occurred exclusively in patients with chronic kidney diseases after ingestion of a mushroom called Sugihiratake. While the exact etiology of this encephalopathy remained mysterious, we aimed to describe its clinical features.

Methods. A total of 32 patients with chronic kidney diseases who had presented with encephalopathy following ingestion of Sugihiratake were enrolled from seven prefectures in Japan., with 24 of the 32 patients undergoing regular hemodialysis. The patient's clinical data were from surveillance by The Japanese Society of Nephrology.

Results. There was a significant association between Sugihiratake ingestion and the occurrence of encephalopathy in 524 hemodialysis patients questioned for a recent ingestion of this mushroom ($P = 0.0006$). The latent asymptomatic period before the onset of symptoms varied from 1 to 31 days (mean 9.1 ± 7.3) days. The patient's symptoms consisted of disturbed consciousness in 30 patients (93.8%), convulsions in 25 (78.1%), myoclonus in 15 (46.9%), dysarthria in ten (31.3%), ataxia in eight (25.0%), paresis or paralysis in seven (21.9%), and skin parasthesia in two patients (6.3%). Nine (27.2%) patients died, mostly due to respiratory failure. The other patients were either discharged or still in hospitals with various degrees of clinical improvement.

Conclusion. Patients with chronic kidney diseases are at risk of having serious encephalopathy following Sugihiratake ingestion and must refrain from eating it. Physicians, in those parts of the world, where this mushroom harvesting is common, should be aware of this complication.

Key words: mushroom intoxication, encephalopathy, chronic kidney disease, hemodialysis.

Received for publication December 11, 2004
and in revised form January 12, 2005
Accepted for publication January 27, 2005

Sugihiratake (Fig. 1) is the Japanese name of the fungus *Pleurocybella porrigens*, which is a small mushroom that grows in abundance during the fall season, not only in the forest of northern Japan, but is also widely distributed across the northern hemisphere [1]. It has an interesting flavor that many Japanese used to enjoy, usually consumed as a component of the highly popular miso (fermented bean paste) soup. Until now, there has been no report of significant adverse effect as a result of Sugihiratake ingestion. However, during the fall of 2004 in Japan, an outbreak of a serious encephalopathy exclusively occurred in patients with chronic kidney diseases after ingestion of this mushroom.

While the exact etiology of the encephalopathy remains mysterious and currently under investigation, we wish to report for the first time the neurotoxic effects of *P. porrigens* mushroom intoxication in a series of Japanese patients with chronic kidney disease.

METHODS

Data used for this study were from surveillance by The Japanese Society of Nephrology. From September through October 2004, a total of 45 patients with chronic kidney diseases presented to several hospitals in eight prefectures in Japan (Fig. 2) because of acute neurologic disturbances. Extensive workup to find out a possible cause was negative other than a history of ingesting Sugihiratake in 44 of the 45 patients during its harvesting season from the end of August through October 2004. Among the 44 patients, 32 (19 females and 13 males) who had their clinical conditions reported to us by the physicians in charge were enrolled in the analysis. Twenty-four patients previously had received regular hemodialysis, whereas eight were not yet undergoing dialysis. The mean age of the patients was 69.2 ± 10.5 years. The underlying kidney diseases were chronic glomerulonephritis in

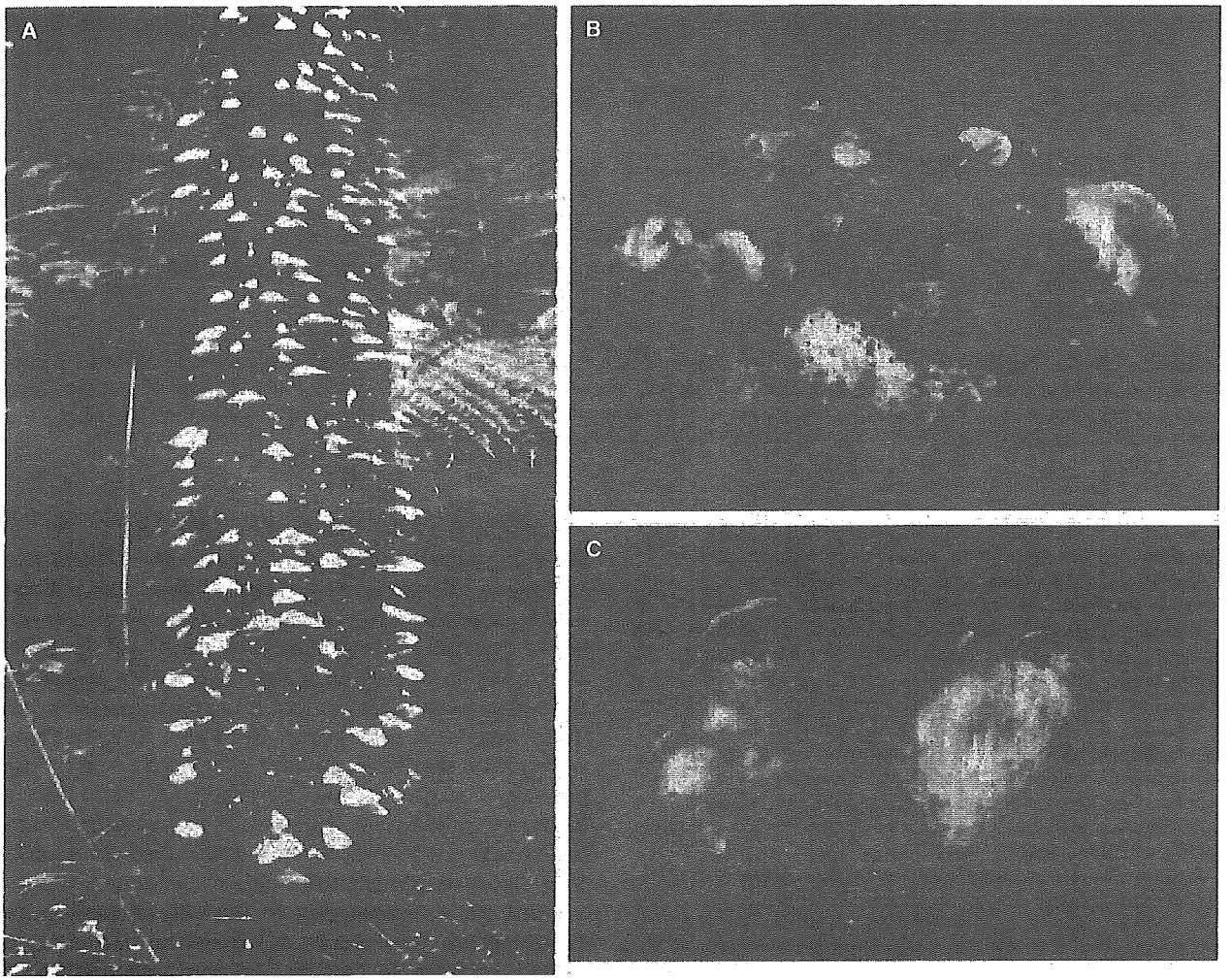

Fig. 1. Photographs of Sugihiratake (*Pleurocybella porrigens*). Sugihiratake have a habitat rooting on woods, especially *Cryptomeria japonica* in Japan forming dense clusters (*A*). The mushroom, which the patient 1 (Table 2) ingested, was that of *P. porrigens* (*B*). The pileus of Sugihiratake, also known as "angel's wings," was 3 to 5 inches long, white in color, and had a wavy margin (*C*).

14 patients, diabetic nephropathy in eight, unknown etiology in five, hypertension in three, and polycystic kidney disease in two patients. All patients, whether dialyzed or not, were in stable clinical condition before this event. We also questioned a total of 524 hemodialysis patients at nine hospitals in the affected area for any recent history of Sugihiratake ingestion and the intoxication rate was calculated. Statistical analyses were conducted by Fisher exact probability test with StatView 5.0 software (Abacus Concepts, Inc., Berkeley, CA, USA).

RESULTS

All cases of acute encephalopathy after Sugihiratake ingestion occurred in patients who had chronic kidney diseases, whereas none of the individuals with normal renal function who had eaten Sugihiratake were affected. Out of 524 hemodialysis patients, 278 (53%) admitted recent ingestion of Sugihiratake, but only 12 patients (4.3%) manifested symptoms of intoxication. By Fisher's exact probability test, there was a highly significant association between the mushroom ingestion by hemodialysis patients and the development of encephalopathy ($P = 0.0006$).

The clinical profile, time of symptom onset, and the outcome are listed in Table 1 for the patients on hemodialysis, and in Table 2 for the patients with pre-end-stage renal disease (pre-ESRD). Nine (28.1%) patients had died so far, five had been uneventfully discharged, and the other 18 patients have remained hospitalized with various degrees of clinical improvement. While many patients continued to eat the mushroom for several days before the symptoms appeared, the time from the start of ingestion to onset of symptoms varied from 1 to 31

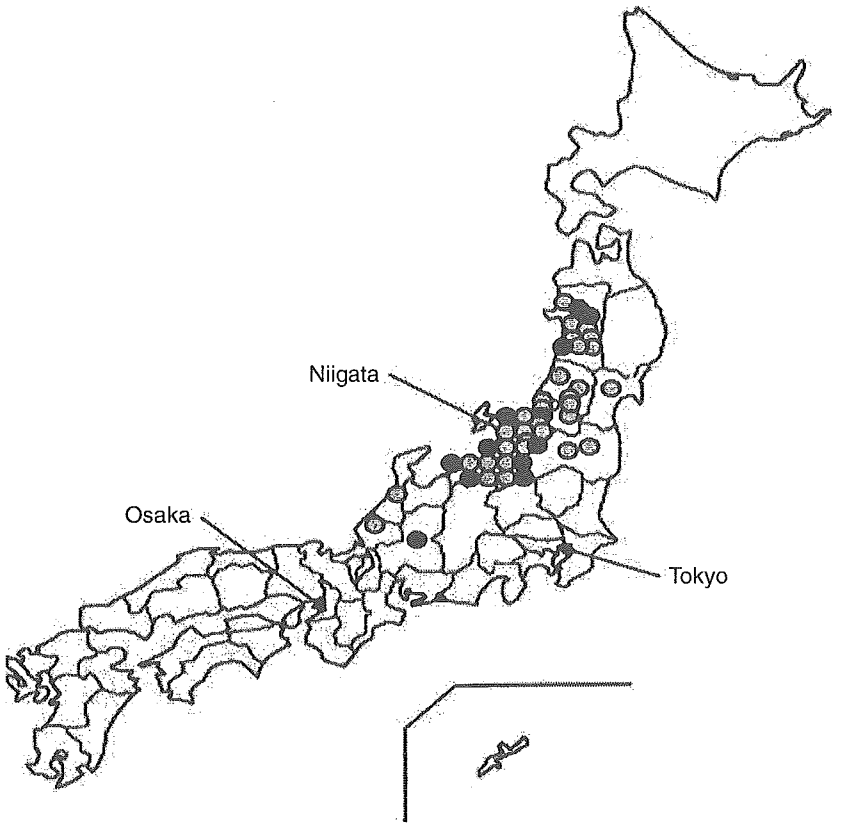

Fig. 2. The distribution of the cases with acute neurological disturbaces. Cases included in the analysis (○) ($N = 32$). Cases not included in the analysis because of insufficient clinical information (●) ($N = 12$).

days (9.1 ± 7.3 days), with no difference between the patients who died or survived. The most common symptoms in all 32 patients included disturbed consciousness of various degrees in 30 patients (93.8%), convulsions in 25 (78.1%), myoclonus in 15 (46.9%), dysarthria in ten (31.3%), ataxia in eight (25.0%), paresis or paralysis in seven (21.9%), and skin parasthesia in two patients (6.3%). Typically, most patients presented initially with ataxia and/or myoclonus several days after Sugihiratake injestion, to be followed in a few days by generalized convulsions. The convulsive activities either stopped without neurologic sequelae or progressed to status epilipticus and death. The terminal event was profound central apnea and bradycardia. Noteworthy, all patients did not experience gastrointestinal symptoms, which are generally common features of mushroom poisoning. Fever was undetectable in almost all patients at the initial presentation; however, some patients had their temperature increased after admission secondary to a respiratory infection.

After being admitted, the patients were closely monitored either in a general ward or in an intensive care unit with the support of a neurologist. The differential diagnosis included metabolic encephalopathy, cerebrovascular accident, viral encephalitis, or possible drug intoxication. Nineteen patients underwent computerized tomographic (CT) brain scans to role out stroke, but no organic lesion could be identified, though in five patients, who ultimately died, brain edema was evident. Lumber puncture was done in ten patients, and the cerebrospinal fluid analysis showed a mild increase of protein content, but normal cell count and sugar. There were no significant changes in the blood biochemistry, including blood sugar, liver function tests, serum aluminum levels, and blood gas analysis compared with the preintoxication levels in most patients.

Treatment depended on the severity of presenting symptoms. Fourteen patients on regular hemodialysis, and two other patients who are not yet on hemodialysis, were assigned to every-other-day dialysis. Ten patients with more severe mental confusion were managed in an intensive care unit with continuous venovenous hemodialysis (CVVHD) and mechanical ventilatory support. Nine patients died 4 to 15 days (mean 8.2 ± 4.1 days) after admission. The principal characteristics of these patients were severe mental confusion in all and convulsive activities in eight.

DISCUSSION

Acute encephalopathy specifically related to patients with uremia may result from any of the following causes; uremic encephalopathy, dialysis dysequilibrium syndrome, cerebrovascular disease, electrolytes

Table 1. Clinical characteristics and outcome of the patients undergoing hemodialysis

Patients	Age	Gender	Duration of hemodialysis *years*	Onset of symptoms *days*[a]	Symptoms[b]	Outcome[c]
1	69	F	5	1	1, 2	Died
2	60	F	8	10	1, 3	In hospital
3	62	F	8	11	1, 2,4, 7	In hospital
4	65	F	8	3	1, 2, 3	In hospital
5	70	F	15	14	1, 2,3, 6	In hospital
6	77	F	5	ND	1, 3, 5	Discharged
7	57	F	5	14	1, 2, 4, 5	In hospital
8	66	F	10	ND	1, 2, 3	In hospital
9	58	F	4	14	1, 2, 6	In hospital
10	60s	F	0.3	12	1, 2, 3	In hospital
11	72	F	2	13	1, 2, 6	In hospital
12	71	F	11	ND	1, 4, 6, 7	In hospital
13	72	F	3	4	1, 2, 4, 6	Died
14	48	F	20	ND	1, 2	Died
15	85	M	1	18	1, 2, 3	In hospital
16	68	M	6	12	1, 2, 4, 5	In hospital
17	78	M	1	31	1, 2, 3	In hospital
18	66	M	3	14	1, 2, 3, 5, 6	Died
19	73	M	1.8	14	1, 2, 3, 4	Died
20	64	M	7	ND	4, 6	Discharged
21	50	M	0.3	1	1, 2, 3	Discharged
22	53	M	16	3	1, 2	Died
23	83	M	1.5	ND	1, 2	Died
24	60	M	2	7	1, 2, 3, 4	In hospital

Abbreviations are: F, female; M, male; ND, not determined.
[a]Time of symptom onset after Sugihiratake ingestion.
[b]1, disturbed consciousness; 2, convulsions; 3, myoclonus; 4, dysarthria; 5, ataxia; 6, paresis/paralysis; 7, parasthesias.
[c]As confirmed by the end of October 2004.

Table 2. Clinical characteristics of the patients with pre-end-stage renal disease (pre-ESRD)

Patients	Age	Gender	Serum creatinine *mg/dL*	Onset of symptoms *days*[a]	Symptoms[b]	Outcome[c]
1	87	F	4.8	3	1, 2, 3, 4, 5	Discharged
2	68	F	5.6	ND	1, 5	Died
3	71	F	5.5	7	1, 2, 5	Died
4	84	F	6.2	2	1, 4	In hospital
5	80	F	1.5	1	1, 2, 3	In hospital
6	89	M	2.0	ND	1, 2, 5	In hospital
7	71	M	4.0	8	1, 2	In hospital
8	67	M	8.0	20	3	Discharged

Abbreviations are: F, female; M, male; ND, not determined
[a]Time of symptom onset after Sugihiratake ingestion.
[b]1, disturbed consciousness; 2, convulsions; 3, myoclonus; 4, dysarthria; 5, ataxia; 6, paresis/paralysis; 7, parasthesias
[c]As confirmed by the end of October 2004.

disorders, and aluminum intoxication [2]. Because of the impaired excretory function, uremic patients are also especially vulnerable to drugs and toxins from many different sources, including food. There have been reports of encephalopathy in uremic patients caused by star fruit (*Averrhoa carambola*) intoxication [3, 4]. However, acute encephalopathy related to mushroom poisoning in uremic patients have not been previously reported.

Two theories for the etiology of this encephalopathy have been speculated. The first theory suggested that an aberrant viral infection, to which uremic patients particularly susceptible, is to be the culprit. However, the absence of family history of similar symptoms, fever, or other markers of acute inflammatory response, in addition to the lack of cerebrospinal fluid pleocytosis stood against this theory. Moreover, the examination for common viral infections was negative. The second theory accused a toxin that is normally metabolized through the kidney. Reviewing the history for a possible intoxication disclosed that almost all of the patients had eaten Sugihiratake. Statistical analysis of hemodialysis patients in the affected area indicated a significant association between Sugihiratake ingestion and the intoxication episodes. Although these findings suggest a causal connection, several questions remained to be answered. If the mushroom is to blame, then, why intoxication occurred this year only,

and why only a small percentage of hemodialysis patients who took the mushroom turned to be symptomatic? What was the nature of this toxin, and how did it cause the encephalopathy?

Despite the fact that wild mushrooms are collected and consumed, becoming poisoned is still a probability. It is well-known that even some experts have difficulty in discriminating between the mushrooms. The extremely wet and hot regional weather in 2004 may have boosted the growth of some poisonous species that are morphologically indistinguishable from the benign mushrooms. In fact, an exceptionally good harvest of Sugihiratake was reported in the affected areas, suggesting that somewhat change in property of the mushroom occurred in this season. In addition, this may support that the amount of ingested mushroom was higher in 2004 than previous years. The toxic characteristics of a mushroom may vary from region to another depending on the soil type [5], and the individual response to certain fungal toxins is also very variable [6]. Taken together, these factors may explain why only limited number of hemodialysis patients were affected.

The connection of the encephalopathy to mushroom poisoning in our series was particularly difficult because of the extraordinary long latent period, and the lack of digestive and hepatic abnormalities, nonetheless, this possibility was worth considered after excluding the other common causes of encephalopathy in uremic patients. In orellanine-containing mushrooms, a latent period of 36 hours to 17 days postingestion is usual before the onset of symptoms, although the clinical symptoms are incompatible [7]. The presentation of mushroom poisoning generally depends on the species and the amount ingested. Encephalopathy was the hallmark of Sugihiratake mushroom poisoning. The fact that hemodialysis was inefficient in preventing the encephalopathy in our series is probably because the elimination capacity was overwhelmed by the amount ingested. Alternatively, it may suggest that the molecular size of the toxin was not small enough to dialyze or that the toxin could have passed the blood-brain barriers, and hence became inaccessible for dialysis. This may be an important contributing factor to its neurotoxic effect. The permeability of the blood brain barrier may have been increased by chemical mediators and cytokines [8]. Experiments to identify and isolate the specific toxin in the mushroom are still undergoing.

In this report, we warn practicing nephrologists that mushroom poisoning must be considered when patients with chronic renal diseases present with conscious disturbances and unexplained neurologic symptoms.

ACKNOWLEDGMENTS

We thank all the physicians in charge, who actively reported to us the clinical progress of their patients: T. Yamagishi (Akita Red Cross Hospital), Y. Morioka (Akita Kumiai General Hospital), M. Suzuki (Yamagata Prefectural Central Hospital), N. Degawa (Nihonkai Hospital), M. Itoh (Okitama Public General Hostipal), H. Igarashi (Sakamachi Hospital), N. Sakurai (Murakami Memorial Hospital), T. Ogihara (Murakami General Hospital), T. Nakatsue (Senami Hospital), Y. Iwahuchi and H. Kobayashi (Sanjyo General Hospital), H. Shimada (Niigata Prefectural Central Hospital), D. Saga (Nanbugo Kosei Hospital), T. Nakamaru (Koide Hospital), T. Takeda (Takeda General Hospital), A. Hamada (Aizu General Hospital), C. Ishida (Noto General Hospital), and M. Hirose (Hirose Hospital). We also thank K. Yokoyama (Shiga University) for invaluable suggestions about mushroom poisoning and N. Matsumoto (Niigata Prefectural Forest Institute) for generously providing the pictures of the mushroom.

Reprint requests to Fumitake Gejyo, M.D., Ph.D., 1-757 Asahimachi-dori, Niigata 951-8510, Japan.
E-mail: gejyo@med.niigata-u.ac.jp

REFERENCES

1. Freedman L, Freedman W: *Wild About Mushrooms* (online edition), San Francisco, Mycological Society of San Francisco, 2004
2. Fraser CL, Arieff AI: Nervous system complications in uremia. *Ann Intern Med* 109:143–153, 1988
3. Chang JM, Hwang SJ, Kuo HT, *et al*: Fatal outcome after ingestion of star fruit (*Averrhoa carambola*) in uremic patients. *Am J Kidney Dis* 35:189–193, 2000
4. Neto MM, da Costa JA, Garcia-Cairasco N, *et al*: Intoxication by star fruit (*Averrhoa carambola*) in 32 uraemic patients: Treatment and outcome. *Nephrol Dial Transplant* 18:120–125, 2003
5. Unluoglu I, Tayfur M: Mushroom poisoning: An analysis of the data between 1996 and 2000. *Eur J Emerg Med* 10:23–26, 2003
6. Karlson-Stiber C, Persson H: Cytotoxic fungi: An overview. *Toxicon* 42:339–349, 2003
7. Jaeger A: Orellanine mushrooms, in *Handbook of Mushroom Poisoning Diagnosis and Treatment*, edited by Rumack BH, Spoerke DG, Boca Raton, FL, CRC Press, 1994, pp 249–264
8. Ballabh P, Braun A, Nedergaard M: The blood-brain barrier: An overview: Structure, regulation, and clinical implications. *Neurobiol Dis* 16:1–13, 2004

Special edition

2 針刺し・切創発生時の倫理的な対応

内山正子　新潟大学医歯学総合病院 感染管理部 感染管理担当看護師長

▶ Summary and Keywords

①針刺し・切創防止のために必要な情報を，医療従事者の目線に立って伝達する．
②当事者に適切な対処が行えるよう，針刺し・切創直後のサポート体制を整える．
③曝露後の対応を行う者は，当事者が自己決定できるよう，情報提供とサポートを行う．
④感染性の有無を確認する検査を行う場合は，患者さんへのインフォームドコンセントを行う．
⑤当事者のプライバシーには十分に配慮する．
⑥報告システムにおける個人情報の取り扱いについて確認する．

針刺し・切創防止の情報提供　サポート体制　プライバシーの保護
個人情報の取り扱い　インフォームドコンセント

はじめに

2005年4月，個人情報保護法が施行されたことにより，医療の中の「倫理的な配慮」について再認識する機会となっている．感染管理担当者も，医療関連感染を取り巻く倫理的な側面について認識し，業務を遂行することが重要である．

私たち医療従事者は「人々の健康の維持・増進を図ること」を使命としており，感染管理担当者は，「患者さんを感染から守る」という視点でこの使命を果たすばかりでなく，「医療従事者を職業感染から守る」という使命もある．その意味で，針刺し・切創発生時の対応は重要である．

表1に，私見ではあるが，針刺し・切創時の倫理的配慮についてのチェックポイントを示した．自施設の現状を評価してみてほしい．

今回は，「医療従事者を職業感染から守る」という側面から，針刺し・切創発生時の倫理的な対応について考えてみたい．

針刺し・切創防止のための情報提供

針刺し・切創を防止するために，各施設でさまざまな取り組みがなされていることと思われる．当施設では，EPINet™（Exposure Prevention Information Network）日本版の，集計・解析ソフトEpisys[1]を活用し分析した結果，「リキャップ」「携帯用針廃棄容器の活用の不徹底」「安全機能付針の不適切な使用」が問題点であることが判

表1 針刺し・切創時の倫理的配慮についてのチェックポイント

①自施設の医療従事者に対して，針刺し・切創を防止するための方法について，適切に情報提供しているか．
②針刺し・切創直後，当事者が曝露後の対応に専念できるサポート体制ができているか．
③曝露後の対応では自己決定が尊重されているか．
④感染症検査を依頼する際，患者さんへのインフォームドコンセントは十分に行われているか．
⑤プライバシーへの配慮がなされているか．
⑥報告システムにおける個人情報の取り扱いは適切か．

明し，それらを解決すべく取り組みを行ってきた．しかし，上記3つによる針刺し・切創は減らない現状にある．

特に，2004年に導入された安全機能付留置針は，操作方法を誤ったことによる医師の針刺し件数が増加した．導入時に各部署の責任者に説明が行われたが，個々の医師の理解を得るまでには至らなかったことが原因の一つと考えられた．そのため，各部署単位で，医師が多く集まる検討会などを利用し，使用方法の説明会を再度実施した．その説明会に参加してみると，実際に使用している医師が正しく使用方法を理解していないという事実が明らかになり，導入時の細やかな情報提供の重要性を認識した．

感染管理担当者は，医療従事者の針刺し・切創による感染リスクや防止方法について，種々の情報を得て学習することで認識している．しかし，学習する前はどうであっただろうか？ 筆者自身，それほど針刺し・切創に意識を向けていなかったように記憶している．その当時の自分と同じ状況にある人々に理解してもらうには，針刺し・切創による感染リスクを正しく認識できるように，また，防止方法を適切に実施できるように，細やかな情報提供と訓練を行うことが重要である．感染管理担当者は，常に，実際に針などを扱う医療従事者の目線に立って対応していくことが望まれる．

針刺し・切創直後のサポート

筆者は以前，針刺し・切創を起こした時，精神的に動揺しながらも業務を中断することができずに，そのまま仕事を続けていたという経験がある．しかしこの時，同僚が，筆者の仕事をほかのスタッフに分担し，曝露後の対応に専念できるよう配慮してくれた．それ以来，自分の周囲で針刺し・切創が発生した場合には，当事者がその対応に専念できるように配慮することを心がけるようになった．このような針刺し・切創発生直後のサポート体制は重要である[2]．

サポートを行う者は，プライバシーに配慮しながら，適切に当事者が対処できるようアドバイスできる身近な存在であることが望ましく，その部署の感染対策担当者（リンクドクターやリンクナースなど）あるいは責任者が適任である．また，担当者が不在の場合や不明な点がある場合には，ICNやICDなどに相談できることも必要で，当事者が「いつでも対応してもらえる」という安心感を得られるような体制が望ましい．曝露後の対応は，その病原体によって対処方法が異なったり，公務災害補償や労務災害補償の申請を含めた院内

の届け出の方法に戸惑ったりする場合が多いため，専門的な知識を持った者が相談窓口となることが適切な対処につながる．

また，当事者を責めるのではなく，支援的態度で接することも重要である．責められることを恐れ報告しないでいることは，当事者に適切な曝露後の対処が行われないばかりでなく，報告を生かした防止方法の検討も行われないことから，施設全体にも不利益が生じる[3]．

自己決定の尊重

針刺し・切創を起こした場合，当事者は自己決定を迫られる場面がある．HBVに曝露した場合は，HBIGという人血液由来の特定生物由来製品を接種するかどうか，HIVの場合は，予防内服を行うかどうか，などである．当事者の自己決定にあたり，対応者は，使用する薬剤の効果や安全性，副作用，相互作用，日常生活上の注意点などについて十分な情報提供を行う．当事者が納得したうえで，対処方法を自己決定できるように支援を行うことが重要である．

日ごろから，針刺し・切創が起こった場合に「自分だったらどうするか」を考えてもらったり，シミュレーションを行ったりする機会を持っておくと，自己決定がスムーズに行える．

患者さんへのインフォームドコンセント

汚染源の感染性が不明な場合は，当該患者さんに依頼し，感染症の有無を明らかにするための抗体検査を実施する．その際，針刺し・切創が起こった事実，検査の必要性，検査の結果の通知方法，得られた情報（検査結果）の取り扱い，費用などについて十分な説明を行い，同意を得る．書式を用意しておくと，患者さんに伝えるべき情報が不足しないであろう．

また，「検査は，陰性であることを確認し当事者が安心するための検査ではない」ということを認識しておかなくてはならない．陰性確認のための検査と認識していたために「感染性がある」と判明して慌てる場合が少なくない．抗体検査の結果が陰性でも患者さんに事実を説明するのはもちろんであるが，陽性であった場合は，新たな疾患の事実を伝えられる患者さんの精神面に配慮しながら説明し，その感染症に対する適切な医療が受けられるよう準備を行う．感染管理担当者は，検査を受ける患者さんへの対応について，あらかじめ確認を行っておくことが必要である．

プライバシーの保護

針刺し・切創に対処する場合，プライバシーの保護については常に配慮する必要がある．私たち医療従事者は患者さんのプライバシーについては十分に配慮しているが，同じ医療従事者，自身のプライバシーに無頓着な場合がある．

相談を受ける場合に，スタッフの多く存在する場所で対応し，針刺し・切創が起こった事実が，必要以上のスタッフにまで知られてしまう場合がある．対応者は，この点に十分配慮し，相談には

個室を使用し，情報の取り扱いには十分に注意する．初期対応を行う部署の担当者や責任者にも，プライバシーの保護についてあらかじめ理解してもらうことが必要である．

また，曝露後，不幸にも感染が成立した場合には，その個人の不利益にならないように，プライバシーへの配慮はもちろんのこと，擁護する存在となることも感染管理担当者の役割である．

報告システムにおける個人情報の取り扱い

多くの施設で,針刺し・切創が起こった場合の，報告書式や報告ルート，書式の活用方法や保管方法が決められていると思われる．個人情報保護法が施行された今，針刺し・切創の報告システムにおいても個人情報の取り扱いについて再考する必要がある．

「施設の現行の報告ルートには本来不必要と思われるルートはないだろうか」「情報の管理方法は適切だろうか」……．病院管理者も含め，感染管理担当者や職員の健康管理担当者と十分な吟味を行い，報告ルートを整理し，情報の管理方法についてもルールを決めておくことが望ましい．

よりよい医療を提供するため，医療従事者を感染から守る

針刺し・切創時の倫理的な対応について述べてきたが，筆者も，感染管理担当者として自分自身がこれまでどうであったかを振り返る機会となった．医療従事者が安全で安心して医療を提供できるよう，「倫理的な配慮」を常に考えながら職業感染防止に努めていきたい．

文　献

1) 職業感染制御研究会ホームページ
http://jrgoicp.umin.ac.jp/index.htm
2) 池田和子．曝露後のサポートとプライバシーの保持の問題．セーフティマネジメントのための針刺し対策A to Z．木戸内清編集，木村哲監修．INFECTION CONTROL 2002年増刊，236-9.
3) Block, JS. 事故報告をどのように扱うべきか．インターナショナルナーシングレビュー．25（4），2002，41-44.
4) 内山正子．針刺し・切創および粘膜曝露による職業感染の管理．INFECTION CONTROL．13（6），2004，23-29.

9

PNA–*In Situ* Hybridization Method for Detection of HIV-1 DNA in Virus-Infected Cells and Subsequent Detection of Cellular and Viral Proteins

Tomoko Hagiwara, Junko Hattori, and Tsuguhiro Kaneda

Summary

We describe *in situ* hybridization protocols using peptide nucleic acid (PNA) as a probe for detecting HIV-1 DNA in virus-infected cells and the subsequent detection of cellular and/or viral proteins. Because a PNA probe of approx 20 bases was sufficiently long to detect a specific target sequence, a conserved sequence of such a short length was easily identified. Therefore, this probe is valuable even to identify quasi-species of HIV-1. In addition, we adopted a catalyzed signal amplification method to amplify weak viral DNA signals; thus, stringent washing was crucial for eliminating false-positive signals. Our double-staining method using PNA–*in situ* hybridization and subsequent immunostaining enabled the active and inactive proviruses to be distinguished.

Key Words: *In situ* hybridization; peptide nucleic acid; catalyzed signal amplification; HIV-1 provirus; CD4-positive T lymphocytes; p24; HLA-DR.

1. Introduction

In situ hybridization (ISH) is now popularly used in cytogenetic studies to determine the localization of a specific gene on a chromosome and to detect mRNA expression and viral infection within cells using DNA or RNA probes. Nonradioactive ISH methods using fluorescence or visible light to visualize signals generally are used for the detection of target nucleic acid sequences. In general, probes of more than 500 bp in length are required in such studies *(1)*.

Until recently, the detection of HIV-1 has been performed by using autoradiography, using probes labeled with radioisotopes, such as ^{35}S *(2–4)* and ^{125}I *(2,5)*. Although the use of radioactive ISH for the detection of HIV-1 is time consuming and not very convenient, the small copy number of HIV-1 in

From: *Methods in Molecular Biology, vol. 326:* In Situ *Hybridization Protocols: Third Edition*
Edited by: I. A. Darby and T. D. Hewitson © Humana Press Inc., Totowa, NJ

infected cells has hindered the development of a conventional nonradioactive detection system. In addition, designing a suitable proper probe with a length of more than 150 bp is very difficult because of the general lack of long-conserved DNA sequences in viruses, such as HIV-1, that have error-prone reverse transcriptase without any repair activity. To bypass this problem, we developed a peptide nucleic acid (PNA) that mimics the DNA configuration ***(6–8)***. PNA, being electrically neutral, can penetrate into cells more easily than DNA and, in addition, PNA can more strongly hybridize with DNA than DNA ***(9,10)***. For these reasons, a PNA probe of approx 20 bases in length is long enough to detect a specific target sequence ***(11)***.

To overcome the weak viral DNA signal, one of the following methods for signal amplification can be used: one is *in situ* polymerase chain reaction (PCR ***[12]***), and the other is a catalyzed signal amplification (CSA) using biotinyl tyramide ***(13)***. *In situ* PCR is quite powerful for amplification of rare target DNA within cells; therefore, *in situ* PCR-driven ISH would be suitable for detecting low copy number DNA sequences. However, this method carries the risk of amplicons synthesized *in situ* diffusing and resulting in false-positives ***(14)***. On the other hand, a single copy of the HPV-16 virus was detected successfully using the CSA method ***(14)***. Therefore, we adopted the CSA method for detecting HIV-1 DNA ***(15)*** and incorporated a crucial stringent washing step to eliminate nonspecific signals that arise from the CSA procedure.

Here, we describe the procedures for detecting HIV-1 DNA in infected CD4-positive T lymphocytes, and the phenotypic determination of HIV-1 DNA-positive cells by a double staining method.

2. Materials

2.1. Cell Lines

1. ACH2: positive control. Human lymphoid cell line latently infected with HIV-1.
2. MOLT4-IIIB: positive control. Human leukemic cell line persistently infected with HIV-1 strain IIIB.
3. MOLT4: negative control.

2.2. Sample Preparation

2.2.1. CD4-Positive T-Lymphocytes Smears

1. StemSep column chromatography (Stem Cell Technologies, Inc., Vancouver, BC, Canada; cat. no. STS-14032).
2. Phosphate-buffered saline (PBS) with 3% fetal bovine serum.
3. 4% paraformaldehyde (PFA) containing 0.1 *M* sodium phosphate buffer, pH 7.4.
4. Ethanol.
5. Silane-coated slides.

6. Wax pen (DakoCytomation A/S, Glostrup, Denmark).
7. Staining racks and containers.
8. Dryer.

2.2.2. Formalin-Fixed, Paraffin-Embedded Tissue Sections

1. Tissue blocks of 20% formalin-fixed or 4% PFA-fixed, paraffin-embedded samples.
2. Silane-coated slides.
3. Staining racks and containers.
4. Xylene.
5. Rehydration series: 100, 95, 90, and 70% ethanol.

2.3. Pretreatment

2.3.1. CD4-Positive T Lymphocytes Smears

1. Tris-buffered saline containing Tween-20 (TBST): 50 m*M* Tris-HCl, 300 m*M* NaCl, 0.1% Tween-20, pH 7.6.
2. Target retrieval solution (DakoCytomation A/S; cat. no. S1700).
3. Methanol containing 0.3% H_2O_2.
4. Water bath.
5. Staining racks and plastic containers.

2.3.2. Formalin-Fixed, Paraffin-Embedded Tissue Sections

1. The same as **Subheading 2.3.1., items 1–5.**
2. Proteinase K (DakoCytomation A/S, S3004).

2.4. Preparation of PNA Probe

1. PNA probe: the structure of the probe is as follows: FITC·HN-GCAGCTTCCT-CATTGATGG-$CONH_2$ (FASMAC Co. Ltd., Kanagawa, Japan; *see* **Note 1**).
2. DNA ISH solution (DakoCytomation A/S; cat. no. S3305).
3. Cover slips.

2.5. Heat Denaturation

1. Hotplate.

2.6. Hybridization

1. Stringent wash solution (DakoCytomation A/S; cat. no. K5201).
2. TBST.
3. Incubator.
4. Plastic containers.
5. Water bath.
6. Moist chamber.

2.7. PNA Probe Detection by CSA

1. Horseradish peroxidase (HRP)-conjugated anti-FITC antibody (DakoCytomation A/S; cat. no. P5100).
2. Biotinyl tyramide solution (DakoCytomation A/S, GenPoint kit K0620).
3. HRP-conjugated streptavidin (DakoCytomation A/S, GenPoint kit K0620).
4. Alexa Fluor 488-labeled streptavidin (Invitogen, Carlsbad, CA).
5. 4,6-diamino-2-phenylindole (DAPI) II (Vysis, Inc., Downers Grove, IL).
6. TBST.
7. Staining racks and containers.
8. Cover slips.

2.8. PNA–ISH and Immunohistochemistry (IHC) (Indirect Method)

1. Mouse anti-human CD4 monoclonal antibody (Novocastra Laboratories, Ltd., Newcastle, UK; cat. no. NCL-CD4-1F6) or mouse anti-human HLA-DR monoclonal antibody (DakoCytomation A/S; cat. no. M0746).
2. Alexa Fluor 594-labeled goat anti-mouse IgG antibody (Invitrogen; cat. no. A-11005).
3. DAPI II (Vysis, Inc.).
4. Cover slips.

2.9. PNA–ISH and IHC (Labeled Streptavidin-Biotin Method)

1. Avidin solution (DakoCytomation A/S; cat. no. X0590).
2. Biotin solution (DakoCytomation A/S; cat. no. X0590).
3. Mouse monoclonal anti-HIV-1 p24 antibody (DakoCytomation A/S; cat. no. M0857).
4. Biotinylated goat anti-mouse Ig antibody (DakoCytomation A/S; cat. no. E0433).
5. Alexa Fluor 594-labeled streptavidin (Molecular Probes, Inc.; cat. no. S-11227).
6. DAPI II (Vysis, Inc.).
7. Cover slips.

3. Methods

3.1. Sample Preparation (see Note 2)

3.1.1. CD4-Positive T Lymphocytes Smears

1. Negatively select and purify CD4-positive T lymphocytes by StemSep column chromatography according to the manufacturer's instructions.
2. Spin down the collected cells at 250*g* for 5 min.
3. Discard supernatant and resuspend with PBS.
4. Mark the area for sample-mount on a silane-coated slide with a wax pen. Drop 5 µL of the cell suspension onto the slide and spread it out gently using the pipet tip.
   The area of the sample-mount (15 × 15 mm).
5. Dry the slides using a dryer at a cool setting.
6. Fix the slides with 4% PFA containing 0.1 mol/L sodium phosphate buffer, pH 7.4, at room temperature for 60 min or at 4°C overnight.

7. Rinse the slides in PBS (3 min, three times).
8. Dehydrate the slides in absolute ethanol and then store at –20°C until use.

3.1.2. Formalin-Fixed, Paraffin-Embedded Tissue Sections

1. Place a 4- to 5-μm section onto a slide. Heat the slide to melt the paraffin in a 60°C oven for 15 min and dry at 37°C overnight.
2. Deparaffinize sections in fresh xylene (3 min, three times) and rehydrate in graded (100, 100, 95, 90, and 70%) ethanols and autoclaved water.

3.2. Pretreatment

3.2.1. CD4-Positive T-Lymphocyte Smears

1. Immerse the slides in autoclaved water for 5 min.
2. Immerse the slides in preheated target retrieval solution for 40 min at 95°C, and allow to cool for 20 min.
3. Wash the slides in autoclaved water (1 min, three times).
4. Immerse the slides in methanol containing 0.3% H_2O_2 for 20 min.
5. Wash the slides in autoclaved water for 1 min.
6. Briefly immerse slides in 95% ethanol and allow to air dry.

3.2.2. Formalin-Fixed, Paraffin-Embedded Tissue Sections

1. Immerse the slides in autoclaved water for 5 min.
2. Immerse the slides in preheated target retrieval solution for 40 min at 95°C and allow to cool for 20 min.
3. Wash the slides in autoclaved water (1 min, three times).
4. Digest sections with proteinase K for 10 min at room temperature (*see* **Note 3**).
5. Wash the slides in autoclaved water (1 min, three times).
6. Treat the slides with methanol containing 0.3% H_2O_2 for 20 min.
7. Wash the slides in autoclaved water for 1 min.
8. Briefly immerse the slides in 95% ethanol and allow to air-dry.
9. Mark the area of the section with a wax pen.

3.3. Preparation of PNA Probe

1. Dilute FITC-conjugated PNA probe in hybridization solution to a final concentration of between 0.2 and 0.5 μg/mL.
2. Apply 25 μL of hybridization solution containing PNA probe to the marked area of the slide.
3. Carefully apply the cover slip, avoiding the introduction of air bubbles.

3.4. Heat Denaturation

1. Heat the slides at 93°C for 5 min on a hotplate to denature the double-stranded DNA.

3.5. Hybridization

1. Incubate the slides with the PNA probe at 45°C for 60–90 min in a moist chamber.
2. After hybridization, immerse the slides in TBST and gently remove the cover slips.

3. Wash the slides in prewarmed stringent wash solution at 57°C (20 min, twice).
4. Immerse the slides in TBST at room temperature for 5 min.

3.6. PNA Probe Detection by CSA (see Notes 4 and 5; Fig. 1)

1. Incubate HRP-conjugated rabbit anti-FITC antibody (1:500 dilution) for 60 min (*see* **Note 6**).
2. Wash the slides in TBST (3 min, three times).
3. Incubate biotinyl-tyramide for 15 min.
4. Wash the slides in TBST (3 min, three times).
5. Incubate HRP-conjugated streptavidin (1:600–800 dilution) for 15 min.
6. Wash the slides in TBST (3 min, three times).
7. Incubate biotinyl-tyramide for 15 min.
8. Wash the slides in TBST (3 min, three times).
9. Incubate 0.5 μg/mL Alexa Fluor 488-labeled streptavidin for 15 min in the absence of light.
10. Wash the slides in TBST in the absence of light (3 min, three times).
11. Immerse the slides in distilled water.
12. Apply DAPI II and mount cover slip.

3.7. Fluorescence Microscopy

The slides were examined under a fluorescence microscope (BX50 and BX-FLA, Olympus Corp., Tokyo, Japan) equipped with appropriate filter sets (61002, Chroma Technology Corp., Rockingham, VT). Photographic images of the fluorescent signals were taken with a CCD camera (SenSys 0400, Photometrics Ltd., Tucson, AZ) and were uploaded to a microcomputer using IPLab software (Scanalytics, Inc., Fairfax, VA). The stored images were merged to reveal various aspects (*see* **Note 7**; **Fig. 2**).

3.8. Subsequent Determination of Phenotypes After PNA–ISH Treatment

We describe here a double staining method by PNA–ISH and IHC methods for the detection of both HIV-1 DNA and a protein in the same cell, respectively. Proteins can be detected with one of two methods with IHC: an indirect method and a labeled streptavidin-biotin (LSAB) method. For the detection of CD4 or HLA-DR molecules, the conventional indirect method was used. On the other hand, the LSAB method that has higher sensitivity than the indirect method was performed to detect the p24 HIV-1 capsid protein.

Fig. 1. (*opposite page*) Schematic representation of the procedures for using the PNA-probe in the ISH method. The hybridized probe was detected by sequential reactions of the following antibodies and reagents: HRP-conjugated anti-FITC antibody, biotinylated tyramide (first amplification), HRP-labeled streptavidin, biotinylated tyramide (second amplification), and streptavidin-conjugated Alexa 488.

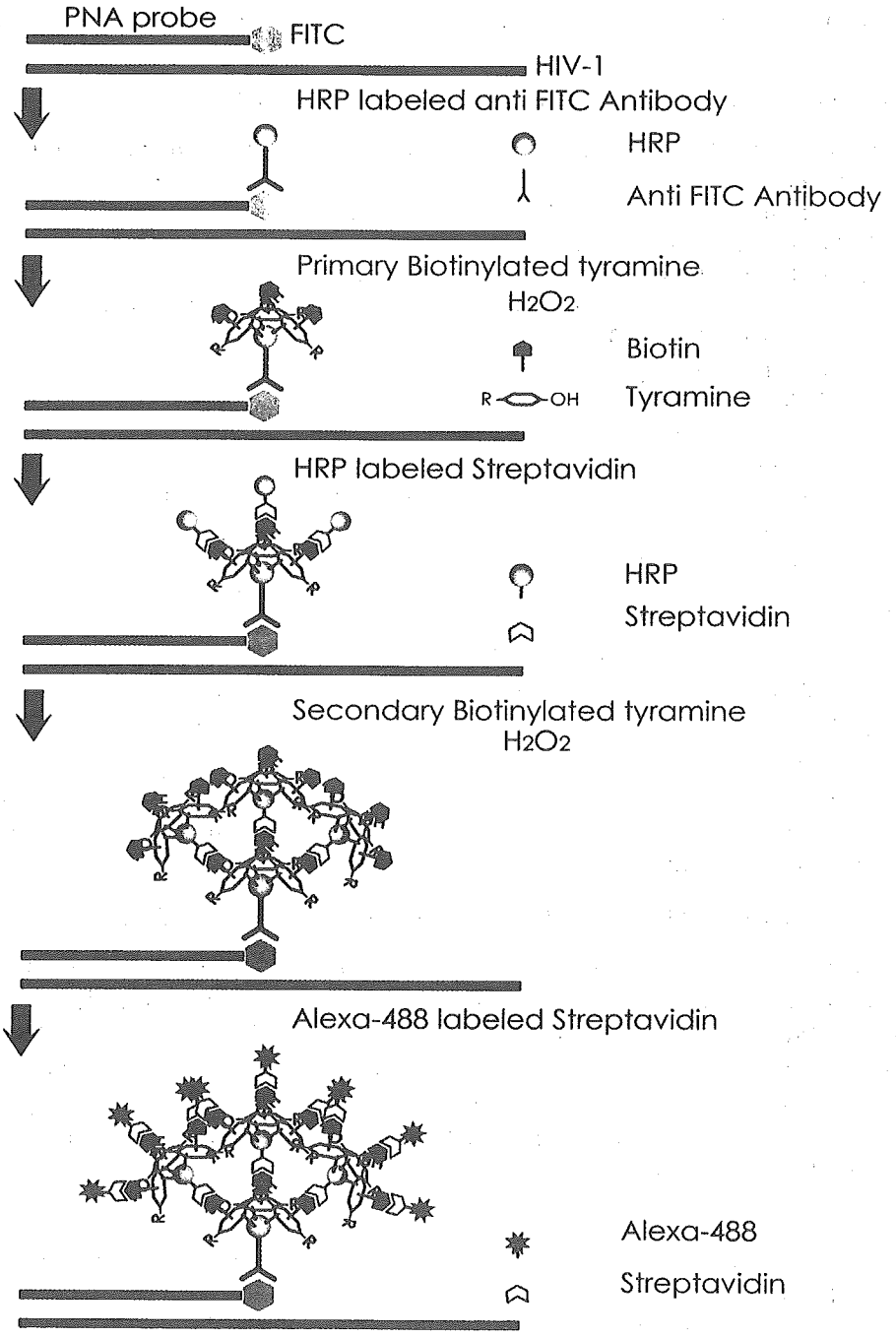
PNA probe
FITC
HIV-1
HRP labeled anti FITC Antibody
HRP
Anti FITC Antibody
Primary Biotinylated tyramine
H2O2
Biotin
R
OH
Tyramine
HRP labeled Streptavidin
HRP
Streptavidin
Secondary Biotinylated tyramine
H2O2
Alexa-488 labeled Streptavidin
Alexa-488
Streptavidin

Fig. 1

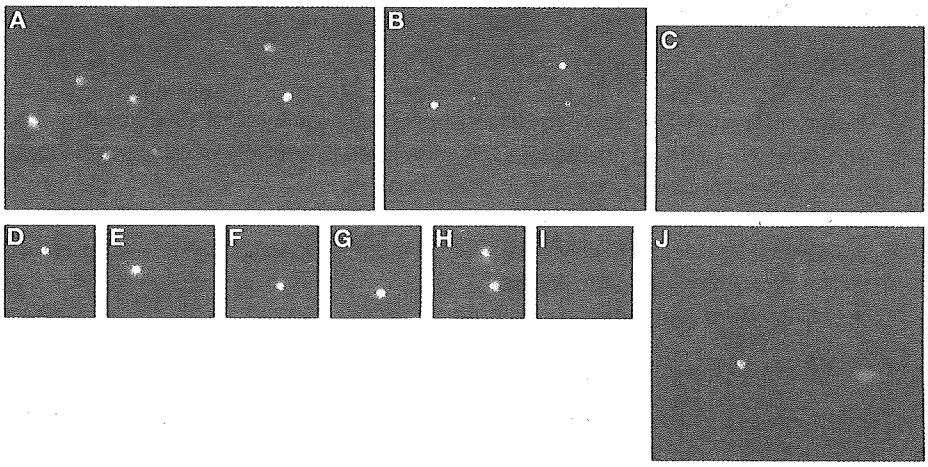

Fig. 2. Detection of HIV-1 provirus by PNA–ISH. Signals detecting HIV-1 proviruses were observed as green dots in a nucleus stained blue by DAPI. (**A**) MOLT4-IIIB. One to four proviruses per cell. (**B**) ACH2. One provirus per cell. (**C**) Negative control (MOLT4). No provirus. (**D–H**) CD4-positive T lymphocytes from HIV-1-infected patients. Most provirus-positive cells contained one provirus. Positive cells rarely contained more than two proviruses. (**I**) Negative control (CD4-positive T lymphocytes from a HIV-1-negative volunteer). No provirus. (**J**) Paraffin-embedded section of bone marrow from an AIDS patient. Positive cells contained one provirus in a nucleus.

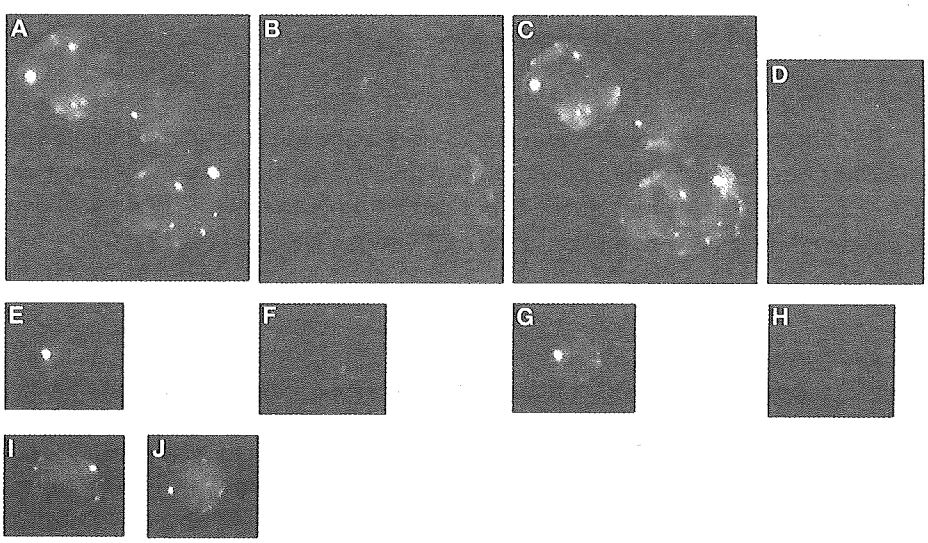

Fig. 3. Subsequent determination of phenotypes after PNA–ISH treatment. Proteins were stained red and localization of the proteins can be observed. (**A–C**) p24/HIV-1 provirus of MOLT4-IIIB. (**D**) Negative control (MOLT4). (**E–G**) p24/provirus