Abstract. Immunotherapy using cytotoxic T-lymphocytes (CTLs) still has limited success. An increase in the frequency of CTL administration is one method to improve immunotherapy using CTLs. The conventional method (C-method) that generates CTLs after the induction of dendritic cells requires a long time period. If CTLs can be more rapidly and simply induced, the frequency of immunotherapy could be increased and unexpected contamination could be avoided. In this study, in order to more rapidly induce functional CTLs, we investigated a new method (N-method) that uses a cytokine cocktail, including interleukin (IL)-2, IL-4, granulocyte macrophage colony-stimulating factor, tumour necrosis factor-α and interferon-α, together with a tumour lysate. CTLs induced by the N-method had equivalent functions, such as proliferation, surface antigen expression and cytotoxicity, compared with those induced by the C-method. These results suggest that the N-method can substitute the C-method in order to improve the effect of immunotherapy using CTLs.

Cytotoxic T-lymphocytes (CTLs) have been successfully used for the treatment of various types of cancers (1, 2). CTLs against tumour cells can be induced by in vitro sensitization of T-lymphocytes to tumour antigens, presented by antigen-presenting cells (APCs) (3, 4). Dendritic cells (DCs) are the most potent APCs and are used for cancer vaccines (5, 6).

Indeed, DCs have a pivotal role in T-lymphocyte-mediated tumour immunity (7, 8). DCs can phagocyte apoptotic and necrotic tumour cells at the tumour site, can migrate to primary lymph nodes, can present tumour-associated antigens (TAAs) to T-lymphocytes and can activate T-lymphocytes by co-stimulatory molecules and secreted interleukin (IL)-12, to induce high numbers of Th1-CD4+ T-lymphocytes and CD8+ CTLs (9). Ada showed that the generation of CD8+ CTLs is more common in a general approach to develop a cancer vaccine followed by the use of DCs as APCs (10).

Conventional antitumour CTLs recognize tumour TAAs presented by major histocompatibility complex (MHC) class I molecules on the surface of tumour cells (11). Behrens showed that CD4+ and CD8+ T-lymphocytes recognize antigens on the same DCs (12). By time-lapse imaging, Pittet showed that tumour-specific CTLs migrate randomly throughout the tumour microenvironment, and sustained migration requires recognition of cognate antigens by CTLs (13).

Recently, many immunological molecular interactions have been clarified, but human tumour cancer immunotherapy has had limited success to date. One of the reasons for this limited success is that tumours can successfully evade immune surveillance. In order to generate effective cellular immunity, one method is to increase the frequency of administration of tumour-specific CTLs. In the present study, in order to improve cell-mediated immunity, we investigated a more rapid method for generating CTLs using a cytokine cocktail including IL-2, IL-4, granulocyte macrophage colony-stimulating factor (GM-CSF), tumour necrosis factor (TNF)-α and interferon (IFN)-α.

Materials and Methods

Generation of CTLs by the conventional method (C-method). Human peripheral blood mononuclear cells (PBMCs; HLA-A2, A24) were maintained in RPMI-1640 medium (Nipro, Osaka, Japan), supplemented with 10% human serum, 100 μg/ml penicillin (Meiji-seika, Tokyo, Japan) and 100 μg/ml streptomycin (Meiji-seika) (hereafter referred to as RPMI medium). Monocyte-derived DCs (Mo-DCs) were generated from the adherent fraction of PBMCs as described elsewhere (14, 15). Briefly, PBMCs were isolated from heparinized peripheral blood by Ficoll/Paque (GE Healthcare Bio-
Sciences Corp., Piscataway, NJ, USA) density gradient centrifugation. PBMCs were resuspended in RPMI medium and allowed to adhere in 6-well culture plates. After 2-h incubation at 37˚C, non-adherent cells were removed and adherent cells were harvested and cultured in RPMI medium. GM-CSF (100 ng/ml; Primune Inc, Kobe, Japan) and 50 U/ml IL-4 (Primimmune Inc) were added to cultures on day 1. On day 7, non-adherent cells were collected as immature Mo-DCs. Then immature Mo-DCs were co-cultured for 24 h with 50 μg/ml of human hepatocellular carcinoma (HCC) Hep-G2 (HLA-A2, A24) cell tumour lysate, prepared by five cycles of freeze/thawing, 3% human serum, 500 U/ml TNF-α (Gentaur Molecular Products, Brussels, Belgium) and 1000 U/ml IFN-α (MSD K.K., Tokyo, Japan) to induce mature Mo-DCs. Autologous lymphocytes were co-cultured with mature Mo-DCs from day 8 to 14. On day 14, lymphocytes were collected and cultured in RPMI medium supplemented with 200 U/ml IL-2 (Primimmune) in 5 μg/ml anti-CD3 monoclonal antibody (OKT3, JANSSEN PHARMACEUTICAL K.K., Tokyo, Japan)-coated culture plates from day 14 to 21. On day 21, lymphocytes were transferred to oxygen permeable culture bags. After an additional seven days’ culture, lymphocytes were collected as CTLs. The total culture time was 28 days. A flow diagram of the process is shown in Figure 1A.

**Generation of CTLs by a new method (N-method).** In order to induce CTLs, human PBMCs (HLA-A2, A24) were cultured in RPMI medium supplemented with 50 μg/ml HepG2 tumour lysate, 3% human serum and a cytokine cocktail including 100 U/ml IL-2, 50 U/ml IL-4, 100 ng/ml GM-CSF, 500 U/ml TNF-α, and 1000 U/ml IFN-α (MSD K.K., Tokyo, Japan)-coated culture plates from day 1 to 7. On day 7, lymphocytes were collected and cultured in RPMI medium supplemented with 200 U/ml IL-2 in 5 μg/ml OKT3-coated culture plates from day 7 to 14. On day 14, lymphocytes were transferred to oxygen permeable culture bags. After an additional seven days’ culture, lymphocytes were collected as CTLs. The total culture time was 21 days. A flow diagram of this process is shown in Figure 1B.

**Flow cytometry.** Surface markers were detected by direct or indirect immunofluorescence using monoclonal antibodies CD3-fluorescein isothiocyanate (FITC), CD4-FITC, CD8-phycoerythrin (PE), CD14-FITC, CD86-PE (Immunotech, Beckman Coulter, Paris, France), NKG2D-PE (R&D System, Minneapolis, MN, USA) and DNAM1-FITC (BioLegend Inc. San Diego, CA, USA). An IgG1 isotype control was also obtained from Beckman Coulter. For staining, cells were incubated with appropriate concentrations of each monoclonal antibody for 1 h at 4˚C. After three washes with phosphate-buffered saline (PBS), the fluorescence intensity was measured by an FC500 flow cytometer (Beckman Coulter) using the FC500 CXP cytometer software (Beckman Coulter).

**Cytotoxicity assay.** We modified an adherent target detachment assay described elsewhere (16), in order to measure the cytotoxicity of
CTLs. Target cells (Hep-G2 cells, 5000 cells/well) were seeded in a 96-well flat-bottom plates and incubated for 24 h to allow adherence. Effector cells (induced CTLs) at an effector:target (E:T) ratio of 20:1 were added to the culture. In some experiments, an anti-MHC class I antibody (W6/32; Abnova Corp., Taipei city, Taiwan) was used to block MHC class I. Target and effector cells were then incubated for 4 h. Dead target cells were detached from the culture surface and were recovered by washing, together with the added effector cells. To quantify viable adherent cells, a WST-8 reagent solution (Dojindo Laboratories, Kumamoto, Japan) was added to the washed wells, followed by incubation for 1 h at 37˚C. The absorbance at 450 nm was then measured using a microplate reader (ImmunoMini NJ-2300; Nalge Nunc International, Rochester, NY, USA).

Statistical analysis. An unpaired two-tailed Student’s t-test was used for statistical analysis. A value of $p<0.05$ was considered significant.

Results

T-lymphocytes successfully contact with monocytes in the N-method. Firstly, we examined T-lymphocyte morphology in the N-method. T-Lymphocytes labelled by immunofluorescence contacted with monocytes and were proliferating on day 2 (Figure 2).

CD14 and CD86 expression in Mo-DCs induced by the N-method is equivalent to that in Mo-DCs induced by the C-method. Firstly, CD14 and representative co-stimulatory molecule CD86 expression in Mo-DCs induced on day 7, was estimated by flow cytometry. CD14 was negative using both methods, suggesting that monocytes successfully differentiated into Mo-DCs. CD86 expression in Mo-DCs induced by the N-method was similar to that in Mo-DCs induced by the C-method (Figure 3).

Proliferation of CTLs induced by the N-method is equivalent to that of CTLs induced by the C-method. Next, proliferation of CTLs induced by the N-method from day 14 to 21 was compared with that of CTLs induced by the C-method from day 21 to 28. There was no significant difference in the proliferation of CTLs between the two methods (Figure 4).

Surface antigens on CTLs induced by the N-method are equivalent to those on CTLs induced by the C-method. Surface antigens such as NKG2D and DNAM-1 on CTLs are molecules that detect tumour cells. Various surface antigens on CTLs induced by the N-method were compared with those on CTLs induced by the C-method. There were no significant differences in surface antigens including natural-
killer group 2 member D (NKG2D), DNAX accessory molecule 1 (DNAM-1), CD3, CD4 and CD8 between CTLs induced by the N-method (on day 21) and those using the C-method (on day 28) (Figure 5).

Cytotoxicity of CTLs induced by the N-method is equivalent to that of CTLs induced by the C-method. Cytotoxicity is the most important factor for CTL-mediated killing of tumour cells. Therefore, we evaluated the cytotoxicity of CTLs induced by both methods. The HLA-A2, A24-matched human HCC cell line, Hep-G2 was used as the target cell line. There was no significant difference in the cytotoxicity of CTLs induced using the N-method (on day 21) and C-method (on day 14) (Figure 6). However, addition of the anti-MHC class I antibody W6/32 resulted in significantly inhibited cytotoxicity of CTLs induced by both methods (Figure 6). This result suggests that CTLs recognized and targeted MHC class I on the tumour cell surface and induced tumour-specific cytotoxicity.

Discussion

Many patients who undergo immunotherapy have disease at a clinically advanced stage, and frequent administration of CTLs is required to treat such patients. Moreover, long culture periods and many steps of operation can lead to contamination. The C-method for CTL induction (in vitro sensitization) involves a long culture period because generation of immature Mo-DCs is required. In our hospital, immunotherapy using CTLs is performed every two to three weeks for most patients. Therefore, the C-method is very problematic because the culture period overlaps with treatment. The N-method used in this study allows for CTLs to be induced at least one week sooner, compared with that of the C-method, and allows many culture steps to be omitted. In addition, the N-method leads to increased therapeutic opportunities and avoids unexpected contamination, and repeated blood collection and apheresis to improve immunotherapy.

We have shown that Mo-DCs were successfully induced by the N-method because the Mo-DC population, gated by forward and side scatter in flow cytometry, were CD14-negative and CD86-positive. We also showed that tumour-specific CTLs were induced by the N-method because cytotoxicity was significantly reduced by the addition of an anti-MHC class I antibody (Figure 6). In the present study, there were no significant differences in the number, the surface antigen expression and the cytotoxicity between CTLs generated using the N-method and C-method, suggesting that enough functional CTLs can be generated using either method. These results suggest that the N-method can substitute the C-method.
In the present study, we used a cytokine cocktail for rapid and simple induction of tumour-specific CTLs. The cocktail contains IL-2, IL-4, IFN-α, TNF-α and GM-CSF. Cytokine cocktails containing IL-2 and IL-4 may be useful for induction and maintenance of CTLs (17). IL-4 can reduce the induction of non-specific cytolytic cells (18). INF-α is a useful cytokine for induction and maturation of antigen-presenting DCs (19, 20). Conventional DCs can be induced by cultivation of monocytes with GM-CSF and IL-4 (21). In the context of *ex vivo*-generated DC-based vaccines, the combination of cytokines used to differentiate monocytes into DCs, might play a critical role in determining the quality of elicited T-lymphocyte responses (22). DCs generated with GM-CSF and IL-15 have the phenotype and characteristics of Langerhans cells and are more efficient at priming melanoma-antigen-specific CD8+ T-lymphocytes *in vitro* than are DCs generated with GM-CSF and IL-4 (23). Using the cytokine cocktail described in this study, we were successfully able to induce CTLs against tumour cells in a short time using a simple process.

In conclusion, using a cytokine cocktail and a tumour lysate, we have successfully induced tumour-specific CTLs. We believe this new method can be used for the rapid induction of CTLs in clinical trials.

**Conflict of Interest Statement**

The Authors declare no conflict of interests.

**References**


