Effect of a Non-Protein Fraction from an Extract of the Inflamed Skin of Rabbits Inoculated with *Vaccinia* Virus (Neurotropin) on Meth A-Induced Delayed Type Hypersensitivity

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Abstract—The effect of a non-protein fraction from an extract of inflamed skin of rabbits inoculated with *vaccinia* virus (Neurotropin, NSP) was studied on Meth A tumor-induced delayed type hypersensitivity (Meth A-DTH) in BALB/c mice. NSP enhanced the Meth A-DTH. NSP also enhanced the DTH suppressed with 5-fluorouracil (5-FU). Moreover, NSP inhibited the fatal effect of 5-FU and restored the decrease of body weight caused by 5-FU. However, NSP reduced partially but significantly the suppression of the tumor growth by 5-FU. NSP may be useful for cancer treatment in combination with chemotherapeutic agents, if NSP does not inhibit their antitumor activity.

Previously, we (1) reported the anti-allergic activity of a non-protein fraction from an extract of the inflamed skin of rabbits inoculated with *vaccinia* virus (Neurotropin, NSP). Yanagihara et al. (2, 3) described that NSP recovered the immune function in mice suppressed by mitomycin C-treatment or irradiation. Yoshii et al. (4, 5) demonstrated that NSP enhanced delayed type hypersensitivity (DTH) against sheep red blood cells in low responder C57BL/6 mice through the induction of Lyt-1+2+ DTH helper T cells, and it also enhanced the DTH in mice suppressed by loading with restraint stress. These findings suggest that NSP has immunostimulating/immunomodulating activity in addition to anti-allergic activity. Recently, we (6) have shown that Meth A-induced tumor-specific delayed type hypersensitivity (Meth A-DTH) can be elicited in BALB/c mice bearing the primary Meth A tumor. In the present paper, the effect of NSP on the Meth A-DTH will be reported. Seven-week old female BALB/c mice were used. They were purchased from SLC Co., Ltd., Shizuoka, Japan. Meth A cells were maintained by weekly passage into the peritoneal cavity of BALB/c mice. Meth A cells for the experiments were collected from the ascites 5–6 days after the i.p. transplantation and washed with Hanks’ balanced salt solution. To study the effect of drugs on the growth of Meth A tumor and Meth A-DTH, a 0.1-ml suspension of $10^6$ Meth A cells was inoculated s.c. into their flanks of BALB/c mice. At 10 days after the inoculation, the size of the Meth A tumor growing subcutaneously was measured using vainer calipers in terms of 2 diameters crossing at right angles. Tumor size was expressed as volume $(4/3\times\pi\times(\text{long diameter}/2)\times(\text{short diameter}/2)^2 \text{ cm}^3)$. The Meth A-DTH was induced by the method described previously (6). Briefly, mice bearing Meth A tumor were challenged by an s.c. injection of a $10^6$ cells/50 µl suspension of mitomycin C (50 µg/ml, 30 min, 37°C)-treated Meth A cells into their right hind footpads. Footpad swelling caused by the challenge was evaluated by the difference in volumes measured just before and 24 hr after the injection with a plethysmometer (model TK-101, Unicom, Yachiyo, Japan). The intensity of DTH was expressed as the difference in footpad swelling between DTH-induced mice and normal mice. NSP was given to the mice i.p. in doses of 5, 10 and 20 mg/kg for 10 consecutive days following the transplantation. Benzylpenicillin potassium-treated and lyophilized cells of the *Streptococcus pyogenes* Su strain (OK-432, Chugai Pharm. Co., Tokyo, Japan) was used.
as a comparative sample at the dose of 0.5 klinische einheit (KE). In an experiment of combined treatment with NSP and 5-fluorouracil (5-FU, Nacalai Tesque, Kyoto, Japan), NSP was given i.p. in doses of 5 and 10 mg/kg and 5-FU was given p.o. in doses of 15 and 20 mg/kg to the mice for 10 days following the transplantation. Meth A-DTH was elicited on day 5, followed by measuring body weight on day 9 and tumor size on day 10. Wilcoxon's rank sum test (U-test) was employed to analyze the significant difference between 2 groups in the data on tumor size. Student's or Welch's t-test after the F-test was used for the statistical analysis of data on parameters other than tumor size. The differences between 2 groups was considered to be significant when P<0.05. NSP was a gift from Nippon Zoki Pharm. Co., Osaka, Japan.

NSP in doses of 5 and 10 mg/kg enhanced the Meth A-DTH significantly, and it tended to enhance the response at the dose of 20 mg/kg (Fig. 1, upper panel). OK-432 also tended to enhance it. Previously, we (6) have reported that OK-432 enhances Meth A-DTH significantly. However, neither NSP nor OK-432 affected the growth of the tumor (Fig. 1, lower panel). OK-432 is known as an immunostimulating agent having host-mediated antitumor activity in animals (7) and humans (8). There are many papers concerning the immunomodulative activity of OK-432, such as activation of macrophages in vivo to kill several lines of tumor cells (9), induction of cytotoxic T-cells in vivo (7), induction of γ-interferon in lymphocyte culture (10) and enhancement of T-cell-mediated immune response in vivo (11). We (12) have described that OK-432 shows a priming activity for tumor necrosis factor (TNF) production in mice, associated with an increase of spleen weight. Spleen cells from BALB/c mice bearing primary Meth A tumor shows neutralizing activity against the tumor (Winn’s assay) (13). A likely explanation for why NSP as well as OK-432 did not suppress the tumor growth even though they potentiated Meth A-DTH is that lymphocytes participating in Meth A-DTH did not migrate into the tumor tissue sufficiently of suppress the growth of Meth A tumor.

Next we examined whether NSP can antagonize the suppressive effect of 5-FU on the antitumor immune response of Meth A-DTH. 5-FU is a well-known potent anticancer agent having immunosuppressive activity. As a preliminary experiment, 5-FU was given to mice p.o. in doses of 10 to 25 mg/kg for 5 or 10 consecutive days after the s.c. transplantation with 10^6 Meth A cells. Meth A-DTH was elicited on day 5 or day 10 following the tumor transplantation, respectively. 5-FU suppressed the Meth A-DTH dose-dependently on day 5, but did not suppress it consistently on
day 10 in repeated experiments (data were not shown). North (14) reviewed cyclophosphamide-sensitive suppressor T cells which down regulate the anti Meth A tumor immune response in BALB/c mice. We have previously reported (6) that Meth A-DTH was obviously elicited in BALB/c mice on day 5, and then the response slightly declined until day 15 and disappeared completely by day 20 in inverse proportion to the growth of the primary tumor. In this case, contact delayed type hypersensitivity of the hosts against picryl chloride was maintained even on day 20, suggesting that the decay of the immune response on day 20 was specific for the Meth A-DTH. On the other hand, in mice immunized with Meth A tumor by excision of the tumor after 7-days growth, a potent Meth A-DTH was observed without any decay of the response at least until day 20 after the excision, and 5-FU (26 mg/kg, p.o., 14 days) abrogated almost completely the Meth A-DTH in the immunized mice (6). The inconsistent suppressive effect of 5-FU on Meth A-DTH on day 10 shown in the present study was probably because suppressor cells might have been generated by day 10, and 5-FU inhibited the suppressor cells. Actually, it is well-known that immuno-suppressive drugs including CY and 5-FU sometimes potentiate anti tumor-immune response. It was, therefore, not suitable for evaluating the suppressive effect of 5-FU on Meth A-DTH 10 days after the transplantation. In the experiment using the combination treatment with NSP and 5-FU, Meth A-DTH was elicited on day 5 after the tumor transplantation. After that, the treatment with drugs was continued until day 9 to evaluate the effect of the combined treatment on the tumor growth, because the tumor size was too small to be measured on day 5.

As shown in Fig. 2 (upper panel), the administration of 10 mg/kg of NSP alone for 5 days showed no significant effect to enhance the DTH elicited on day 5 in contrast to the results of Fig. 1 showing that the administration of 5 and 10 mg/kg of the drug for 10 days enhanced the DTH elicited on day 10 significantly. The treatment with NSP for 5 days may be too short to enhance the response. The administration of 5-FU alone suppressed the DTH elicited on day 5 dose-dependently. The response of the group treated with 25 mg/kg of 5-FU was significantly less than that of the non-treated group at P<0.05. The combined treatment with 5 mg/kg of NSP enhanced significantly the DTH suppressed with 15 and 20 mg/kg of 5-FU, and the combined treatment with 10 mg/kg of NSP showed no significant effect to enhance the DTH suppressed with 15 mg/kg of 5-FU. However, both doses of NSP did not restore the DTH suppressed with the highest dose of 5-FU (25 mg/kg). As indicated in Fig. 2, 4 mice out of 8 died by day 11 in the group treated with 25 mg/kg of 5-FU alone (one mouse died on day 9 and 3 on day 10). However, no mice died in the group treated with 25 mg/kg of 5-FU in combination with 5 mg/kg of NSP. In the group of combined treatment with 25 mg/kg of 5-FU and 10 mg/kg of NSP, only one mouse out of 8 died on day 11. The body weight of the mice was decreased by the treatment with 5-FU dose-dependently. The mean body weights of groups treated with 20 and 25 mg/kg of 5-FU were significantly smaller compared with that of the non-treated group at P<0.01. NSP restored partially but significantly the weight decreased by the treatment with 20 and 25 mg/kg of 5-FU (Fig. 2, middle panel). 5-FU suppressed the tumor growth dose-dependently and significantly in all doses at P<0.01. NSP reduced partially but significantly the suppressive effect of 20 and 25 mg/kg of 5-FU on the tumor growth (Fig. 2, lower panel).

These results suggest that NSP does not only enhance the Meth A-DTH suppressed by 5-FU but also prevents the fatal effect of 5-FU, although the mechanism of the effect of NSP is not known. It, however, seems likely that NSP improves the general body condition damaged by 5-FU through the recovery of the suppressed immunity. Yoshii et al. (15) have described that long-term administration of NSP restores the decreasing T-cell dependent immune responses through the recovery of IL-2 and in part IL-1 production in aging BALB/c mice. In addition to the anti-tumor activity of 5-FU, there is a possibility that a decrease of body weight and the damaged body condition by the treatment with 5-FU (20 and 25 mg/kg) cause the delay of tumor growth. The NSP-induced improve-
ment of general body condition from the damage by 5-FU may result in the prevention of the delay of the tumor growth owing to the body condition impaired by 5-FU. Further investigation is needed to determine whether NSP has an essentially antagonistic action against the antitumor activity of 5-FU or not. It also remains unclear if the activities of NSP to enhance Meth A-DTH and to recover the response suppressed by 5-FU occur through the same immunological mechanism or not.

In conclusion, NSP potentiated Meth A-DTH in mice bearing the tumor as well as that suppressed by the antitumor agent 5-FU. Chemotherapeutic agents including 5-FU and cyclophosphamide generally exhibit the side effect of suppressing immune responses. It is widely accepted that immune responses play a role in host defense mechanisms against tumors. Therefore, the immunosuppression caused by chemotherapeutic agents causes serious side effects in patients undergoing cancer therapy. In this respect, NSP may be useful for the immunotherapy of cancer not only alone but also in combination with immunosuppressive chemotherapeutic agents, if NSP does not antagonize their antitumor activity.

Fig. 2. Effect of NSP, 5-FU and their combined treatment on Meth A-DTH, mortality, body weight and tumor size in BALB/c mice bearing the primary tumor. Eight mice in each group were transplanted s.c. with 10⁶ Meth A cells into their flanks on day 0. NSP was given i.p. and 5-FU was given p.o. for 10 consecutive days from day 0. Meth A-DTH was elicited on day 5. Body weight was measured on day 9. Tumor size was measured on day 10. *: Number of surviving animals on day 11. Each column represents the mean±S.E. **: Statistically significant difference from the group treated with 5-FU alone at P<0.05 and P<0.01, respectively.
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References


