Novel Low Immunosuppressive Derivatives of the Antitumor Drug Fluoropyrimidine, UK-21 and UK-25: Effect on Delayed Type Hypersensitivity and Tumor Immunity

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ABSTRACT—Previously, we reported that two novel 5-fluoropyrimidine derivatives, 2',3',5'-tris-O-[N-(2-n-propyl-n-pentanoyl)glycyl]-5-fluorouridine (UK-21) and 1-{6-[N-(2-n-propyl-n-pentanoyl)glycyl]amino-n-hexylcarbamoyl}-5-fluorouracil (UK-25), show potent antitumor activity with low immunotoxicological effects. The purpose of this paper was to evaluate the effect of these drugs on delayed type hypersensitivity (DTH). Not only UK-21 and UK-25 but also tegafur (FT-207) and 5-fluorouracil (5-FU) produced no suppression of picryl chloride (PC)-induced DTH in mice but rather enhanced it. It is known that variation of the sensitizing antigen dose alters the effect of drugs on the immune response. Because it was difficult to control the antigen dose in PC-DTH, the sheep erythrocyte (SRBC)-induced response was used to examine the effect of drugs on delayed type hypersensitivity in the succeeding experiments. Either a therapeutic dose or an over-dose of the respective drug was given to mice sensitized with 5 $\times$ 10$^5$ or 5 $\times$ 10$^7$ SRBC. The suppressive effects of UK-21 and UK-25 on the DTH were lower than those of FT-207 and 5-FU. UK-21 and UK-25 enhanced Meth A tumor-specific DTH in BALB/c mice, but FT-207 and 5-FU did not. UK-21, UK-25 and FT-207 showed a tendency to enhance or restore the Meth A tumor neutralizing activity of spleen cells in mice bearing the tumor, but carmofur (HCFU) did not. These results indicated that the suppressive effects of UK-21 and UK-25 on the tumor immune response were also low.

Keywords: 5-Fluoropyrimidine derivatives (UK-21 and UK-25), Immunotoxicity, Delayed type hypersensitivity, Erythrocyte (sheep), Meth A tumor

It is well known that antitumor drugs such as antimetabolites and alkylating agents cause various kinds of side effects including immunosuppression, bone marrow injury and digestive system dysfunction. The immunosuppression and injury of the bone marrow by the drugs will reduce not only immunological defense functions against microorganisms but also immunological resistance against tumors. Such suppressive activities of antitumor drugs are paradoxical and are serious side effects from the viewpoint of the therapeutic efficacy of drugs.

Previously, we reported that $\alpha$-mercaptopropionylglycine and sodium dipropylacetate showed antitumor action through their immunostimulating activity (1-4), and their related compound, (2-n-propyl-n-pentanoyl)glycine (KN-539), had host-dependent antitumor activity (5). Thereafter, we (6) found that 2',3',5'-tris-O-[N-(2-n-propyl-n-pentanoyl)glycyl]-5-fluorouridine (UK-21), a conjugate of 5-fluorouridine (5-FUR) and KN-539, and 1-{6-[N-(2-n-propyl-n-pentanoyl)glycyl]amino-n-hexylcarbamoyl}-5-fluorouracil (UK-25), a conjugate of 5-fluorouracil (5-FU) and KN-539 (Fig. 1), showed potent...
antitumor activities with lower immunosuppressive side effects: 1) UK-21 and UK-25 suppressed the growth of Meth A and EL4 tumors in the corresponding syngeneic hosts without decreasing body weight and blood leukocyte count. 2) UK-21 and UK-25 seemed to express their antitumor activity as 5-FUR and 5-FU, respectively. 3) Neither UK-21 nor UK-25 suppressed thymus weight and humoral antibody production against sheep red blood cells (SRBC) in mice, although 1-(2'-tetrahydrofuryl)-5-fluorouracil (FT-207, tegafur) and 5-FU suppressed them in their respective therapeutic dose ranges for the tumors. Thus, UK-21 and UK-25 were expected to develop into anticancer drugs with lower immunotoxicological effects.

The purpose of this paper was to study the effect of UK-21 and UK-25 on the induction of delayed type hypersensitivity (DTH) in comparison with those of their related antitumor fluoropyrimidine derivatives.

First, we examined the effects of the drugs on picryl chloride and SRBC-induced DTH in mice. The complexities and difficulties in evaluating the immunosuppressive potencies of the drugs and the preferable way to perform the evaluation will also be discussed.

It is generally accepted that T lymphocytes primed to tumor-associated antigens play an important role in host defense mechanisms against tumors (7, 8) in addition to the involvement of some other effector mechanisms including natural killer (NK) cells (9), antibody dependent cell-mediated cytotoxicity (10) and cytotoxic antibodies (11). It has been also pointed out that not only Lyt-1-2+ T cells (12-14) including cytotoxic T lymphocytes but also Lyt-1-2- (L3T4+) T cells (12, 15, 16) including delayed type hypersensitivity effector cells and amplifier/helper cells play a role in the host defense mechanisms against tumors. Previously, we (17) reported that Meth A tumor-specific delayed type hypersensitivity (Meth A-DTH) could be detected in BALB/c mice bearing the primary tumor. UK-21 and UK-25 and the other antitumor drugs were examined for their effect on this Meth A-DTH reaction.

Finally, we examined the effect of UK-21, UK-25 and the other drugs on Meth A tumor-neutralizing activity of spleen cells in mice bearing the primary tumor to discuss the suppressive and/or stimulative activities of the drugs against the tumor-immune response.

**MATERIALS AND METHODS**

**Drugs**

UK-21 (M.W. 812), UK-25 (M.W. 405) and 1-hexylcarbamoyl-5-fluorouracil (HCFU, carmofur, M.W. 257) were synthesized at the Medical Research Institute of Ube Industries, Ltd. (Ube). 1-(2'-Tetrahydrofuryl)-5-fluorouracil (FT-207, tegafur, M.W. 200) was a gift from Taiho Pharmaceutical Industry Co., Ltd., Tokushima. 5-Fluorouracil (5-FU, M.W. 130) and cyclophosphamide (CY, M.W. 278) were purchased from Nacalai Tesque, Inc., Kyoto. These drugs were suspended or dissolved in ethanol and mixed with olive oil to give a 5% ethanol solution for oral administration immediately before usage.

**Animals**

Male BALB/c mice and male and female ddY mice (Japan SLC, Inc., Hamamatsu) were used at 7-8 weeks of age. They were maintained with free access to pellet food and water in filtered laminar air flow isolation cages at 21±1°C temperature and 60% humidity.

**Picryl chloride-induced delayed type hypersensitivity (PC-DTH)**

Mice were sensitized by painting 0.1 ml of 1% PC dissolved in ethanol on the shaved skin of their abdomens and challenged 6 days later by painting 15 μl of 1% PC in olive oil on each face of both ear lobes. The PC-DTH intensity was evaluated by the difference of ear thicknesses measured just before and 24 hr after the challenge. A dial thickness gauge (Ozaki Mfg. Co., Ltd., Tokyo) was used for the measurements.

**SRBC-induced delayed type hypersensitivity (SRBC-DTH)**

SRBC stored in Alsever's solution was aseptically washed well with ethylenediaminetetraacetate (EDTA)-gelatin veronal buffer (pH 7.4), gelatin veronal buffer containing Ca++ and Mg++ (pH 7.4), and saline in this order by centrifugation, and resuspended into saline. Female ddY mice were sensitized by an s.c.-injection of an appropriate number of SRBC/40 μl into their left hind foot pads and then challenged by an s.c.-injection of 10^6/40 μl SRBC into their right hind foot pads 5 days after the sensitization. The SRBC-DTH intensity was evaluated as the difference in volumes of the left and right foot pads measured 24 hr after the challenge by a plethysmometer (model TK-101, Unicom Co., Yachiyo).

**Meth A tumor**

Meth A tumor cells were maintained by weekly passage in the peritoneal cavity of BALB/c mice and were collected from the ascitic fluid by centrifugation followed by washing with Hanks' balanced salt solution (HBSS).

**Meth A tumor-induced delayed type hypersensitivity (Meth A-DTH)**

One million Meth A cells/0.1 ml were inoculated s.c. into the flanks of BALB/c mice. The size of the tumor growing in the subcutis was determined with vernier calipers in terms of 2 diameters at a right angle and ex-
pressed as a volume (cm³), which was calculated as follows:

\[
\frac{4}{3} \pi \times \left(\frac{\text{long diameter}}{2}\right) \times \left(\frac{\text{short diameter}}{2}\right)^2
\]

Meth A-DTH was elicited as described previously (17). Briefly, the mice were challenged twice at 10 and 20 days after the transplantation by an s.c.-injection of \(10^6\) mitomycin C (MMC)-treated Meth A cells/50 μl into their right hind footpads. Meth A cells used for the challenge were treated with 50 μg/ml of MMC at 37°C for 30 min in HBSS, then washed three times and resuspended with HBSS. Footpad swelling by the injection of MMC-treated Meth A cells was calculated as the difference in volumes measured just before and 24 hr after the challenge by a plethysmometer (model TK-101, Unicom). Non-specific footpad swelling in normal mice by the s.c.-injection of MMC-treated Meth A cells was also measured and the mean value (among 4 and 10 μl) was subtracted from those of tumor bearing mice to express the Meth A-DTH intensity.

Meth A tumor-neutralizing activity of spleen cells

One million Meth A cells were inoculated s.c. into the flanks of BALB/c mice. They were killed under ether anesthesia 10 and 20 days after the inoculation to provide spleen cells for examining their neutralizing activity against Meth A cells by Winn’s assay. Spleen cell suspension was made as follows: Briefly, spleens were aseptically taken from mice and then gently crushed and separated into single cells by squeezing in HBSS containing 5 U/ml heparin. The cells were passed through a fine stainless steel sieve (200 mesh), washed twice and resuspended in HBSS. The spleen cells obtained from 4 mice per group were pooled and used for the assay. Ten million spleen cells were mixed with \(10^5\) Meth A cells. The mixture was inoculated s.c. into the flanks of 5 to 6 normal BALB/c mice. Tumor size was measured 15 days after the inoculation by using vernier calipers and calculated as described above. The neutralizing activity of the spleen cells was represented as percentage inhibition of the tumor size against that of the control mice, which had been inoculated with the mixture of normal spleen cells and Meth A cells.

Statistics

For all data, the values were expressed as the mean ± S.E.M. Wilcoxon’s rank sum test (U-test) was employed to analyze the statistical difference between two groups in the data on tumor size. Either Student’s two tailed t-test or Welch’s two tailed t-test after the F-test was used for the other data. The value P < 0.05 was considered to indicate a significant difference.

RESULTS

Effect of the drugs on PC-DTH

UK-21, FT-207 and 5-FU were given for 9 consecutive days from 3 days before the sensitization until the day before the induction of PC-DTH. Thymus and spleen weights were measured immediately after the PC-DTH assay. None of the examined drugs suppressed the PC-DTH (Table 1). UK-21 at the dose of 0.2 mmole/kg and 5-FU at the dose of 0.1 mmole/kg enhanced the reaction. FT-207 at 0.4 mmole/kg and 5-FU at 0.2 mmole/kg tended to enhance it. Thymus weights decreased significantly at 0.8 mmole/kg of FT-207 and 0.2 mmole/kg of 5-FU. Spleen weight was not affected by any drug (data not shown).

Table 1. Effect of UK-21, FT-207 and 5-FU on picryl chloride-induced delayed type hypersensitivity (PC-DTH) and thymus weight in ddY mice

| mmole/kg | N  | PC-DTH \times 10^{-3} mm (%) | Thymus weight mg (%) |
|----------|----|---------------------------|---------------------|
| Control  | 8  | 8.3±0.9 (100)             | 38±2 (100)          |
| UK-21    | 0.05 | 7 | 9.5±1.5 (114)          | 41±4 (108)          |
|          | 0.1 | 8 | 8.2±0.7 (98)           | 33±3 (87)           |
|          | 0.2 | 8 | 14.8±3.3* (177)        | 36±3 (95)           |
| FT-207   | 0.2 | 8 | 9.9±1.8 (119)          | 34±4 (89)           |
|          | 0.4 | 8 | 12.4±2.7 (148)         | 32±3 (84)           |
|          | 0.8 | 8 | 7.9±0.7 (95)           | 10±1* (25)          |
| 5-FU     | 0.1 | 8 | 17.1±2.8* (205)        | 40±5 (105)          |
|          | 0.2 | 8 | 13.4±2.5 (161)         | 26±3* (69)          |

N: Number of mice. Mice were sensitized by painting PC on their abdomen and then challenged on their ears 6 days later. Drugs were given p.o. for 9 days starting 3 days before the sensitization. Data represent means±S.E. *: Statistically significant difference from the control at P<0.05 and P<0.01, respectively.

Table 2. Effect of UK-25, HCFU, FT-207 and 5-FU on picryl chloride-induced delayed type hypersensitivity (PC-DTH) and thymus weight in ddY mice

| mmole/kg | N  | PC-DTH \times 10^{-3} mm (%) | Thymus weight mg (%) |
|----------|----|---------------------------|---------------------|
| Control  | 8  | 9.4±2.0 (100)             | 40±4 (100)          |
| UK-25    | 0.1 | 8 | 8.7±1.6 (92)             | 36±4 (89)           |
|          | 0.2 | 8 | 9.9±1.3 (106)            | 39±5 (98)           |
|          | 0.4 | 8 | 13.8±2.0 (147)           | 32±3 (80)           |
| HCFU     | 0.1 | 8 | 12.6±2.2 (134)           | 40±2 (99)           |
|          | 0.2 | 7 | 6.0±0.6* (64)            | 18±1* (45)          |
|          | 0.4 | 8 | 4.6±0.6* (49)            | 9±1* (22)           |
| FT-207   | 0.2 | 8 | 13.9±2.7 (149)           | 40±2 (99)           |
|          | 0.4 | 8 | 11.2±1.7 (120)           | 23±4* (58)          |
|          | 0.8 | 8 | 10.3±2.5 (110)           | 10±1* (25)          |
| 5-FU     | 0.1 | 8 | 11.6±2.2 (124)           | 38±3 (94)           |
|          | 0.2 | 6 | 13.1±2.9 (140)           | 31±3 (77)           |

See legend to Table 1 for details.
The effect of UK-25 on the PC-DTH was also examined in comparison with those of HCFU, FT-207 and 5-FU (Table 2). These drugs were given in the same manner as described above. UK-25 did not suppress PC-DTH at any dose, but showed a tendency to enhance it at 0.4 mmole/kg. UK-25 had little effect on thymus weight. On the other hand, HCFU suppressed PC-DTH significantly at doses of 0.2 and 0.4 mmole/kg in association with a significant decrease of thymus weight. FT-207 and 5-FU showed no suppression of PC-DTH but had a tendency to enhance it, although FT-207 decreased the thymus weight at 0.4 and 0.8 mmole/kg, and 5-FU showed a tendency to decrease the weight at 0.2 mmole/kg. Again, spleen weight was not much affected by any of the drugs (data not shown).

**Effect of the drugs on SRBC-DTH**

As a preliminary experiment, mice were sensitized with varying doses of $1 \times 10^5 - 5 \times 10^6$ SRBC and then challenged with $1 \times 10^8$ SRBC. Some of the mice were given CY at a dose of 150 mg/kg, i.p. at 3 days before the sensitization (Fig. 2). The SRBC-DTH intensity in the CY non-treated control mice increased dependently on the SRBC dose for sensitization, and it reached a peak by sensitization with $10^8$ SRBC. The intensity in mice treated with CY and sensitized with $10^7$ or $10^8$ SRBC was not different from that of the control mice. However, in mice treated with CY and immunized by $10^7$ or more SRBC, the intensity was 2–2.5 times that of the control mice. In the following experiments, doses of $5 \times 10^5$ and $5 \times 10^7$ SRBC were used for the sensitization, and the respective responses were referred to as $5 \times 10^5$ SRBC-DTH and $5 \times 10^7$ SRBC-DTH, respectively.

**Fig. 2. Effect of cyclophosphamide (CY) on delayed type hypersensitivity (DTH) to sheep red blood cells (SRBC) in ddY mice.** Mice were sensitized with various doses of SRBC, s.c. into the left hind footpad and then challenged 5 days later by an s.c.-injection of $10^8$ SRBC into the right hind footpad. CY was given i.p. at a dose of 150 mg/kg 3 days before the sensitization. The DTH intensity was evaluated by the increase of the footpad volume 24 hr after the challenge. Points represent means ± S.E. of 8 mice. ◦: Treated with CY. †: Statistically significant difference from the control at $P<0.01$.

FT-207 or 5-FU was given to mice for 5 consecutive days starting the day of sensitization (Table 3). FT-207 did not affect $5 \times 10^5$ SRBC-DTH at 0.4 mmole/kg and showed a tendency to suppress it at 0.8 mmole/kg. On the other hand, FT-207 enhanced $5 \times 10^7$ SRBC-DTH significantly at both doses. The results for 5-FU resembled those for FT-207. 5-FU at 0.4 mmole/kg showed a tend-

| Sensitized with                               | $5 \times 10^5$ SRBC | $5 \times 10^7$ SRBC |
|-----------------------------------------------|-----------------------|-----------------------|
|                                              | DTH intensity (%)     | Thymus weight mg (%)  | DTH intensity (%)     | Thymus weight mg (%)  |
|                                              | ml                    |                       | ml                    |                       |
| Control                                      | 27.2±4.0 (100)        | 62±4 (100)            | 31.4±4.1 (100)        | 53±4 (100)            |
| FT-207                                       | 28.2±4.2 (104)        | 49±4* (79)            | 69.6±6.2* (222)       | 45±5 (85)             |
|                                              | 21.7±4.6 (80)         | 22±3* (35)           | 47.9±5.9* (152)       | 22±1* (41)           |
| Control                                      | 27.3±4.7 (100)        | 65±6 (100)            | 35.0±3.8 (100)        | 64±3 (100)            |
| 5-FU                                         | 34.1±4.6 (125)        | 37±4* (57)            | 53.0±4.2* (151)       | 45±5* (70)            |
|                                              | 17.6±5.6 (65)         | 19±1* (30)           | 43.0±9.4* (123)       | 19±1* (29)           |

Mice were sensitized with $5 \times 10^5$ or $5 \times 10^7$ SRBC, s.c. into the left hind footpad and then challenged 5 days later by an s.c.-injection of $10^8$ SRBC into the right hind footpad. Drugs were given p.o. for 5 days starting the day of sensitization. The DTH intensity was evaluated by the increase of the footpad volume 24 hr after the challenge and then the thymus weight was measured. Data represent means ± S.E. of 7 or 8 mice. *, †: Statistically significant difference from the control at $P<0.05$ and $P<0.01$, respectively.
Fig. 3. Effect of therapeutic doses of UK-21, UK-25, FT-207 and 5-FU on sheep red blood cells-induced delayed type hypersensitivity (SRBC-DTH) in ddY mice. Mice were sensitized with $5 \times 10^5$ or $5 \times 10^7$ SRBC into the left hind footpad and then challenged 5 days later by an s.c.-injection of $10^8$ SRBC into the right hind footpad. Drugs were given p.o. for 5 days starting the day of sensitization. The DTH intensity was evaluated by the increase of the footpad volume 24 hr after the challenge. Columns represent means ± S.E. of 7 or 8 mice. *: Statistically significant difference from the control at $P<0.05$ and $P<0.01$, respectively. ND: Not done.

Table 4. Effect of therapeutic doses of UK-21, UK-25, FT-207 and 5-FU on thymus weight in ddY mice sensitized with sheep red blood cells (SRBC)

| Sensitized with | $5 \times 10^5$ SRBC | $5 \times 10^7$ SRBC |
|-----------------|----------------------|----------------------|
| mmole/kg        | Thymus weight mg (%) | Thymus weight mg (%) |
| Control         | 52 ± 4 (100)         | 52 ± 3 (100)         |
| UK-21           | 0.1 64 ± 3 (124)     | 55 ± 5 (105)         |
|                 | 0.2 59 ± 3 (113)     | 56 ± 3 (109)         |
|                 | 0.4 67 ± 6 (129)     | 40 ± 7 (77)          |
| UK-25           | 0.2 64 ± 4 * (124)   | 54 ± 4 (105)         |
|                 | 0.4 48 ± 5 * (92)    | 48 ± 4 (92)          |
|                 | 0.6 47 ± 3 (90)      | 24 ± 3 1 (46)        |
| FT-207          | 0.2 55 ± 8 (106)     | 54 ± 5 (104)         |
|                 | 0.4 39 ± 3 * (75)    | 40 ± 7 (78)          |
| 5-FU            | 0.1 64 ± 5 (123)     | ND                   |
|                 | 0.2 45 ± 3 (87)      | 32 ± 5 1 (62)        |

See legend to Fig. 3 for details. The thymus weight was measured after the SRBC-DTH assay. Data represent means ± S.E. of 6 to 8 mice. *: Statistically significant difference from the control at $P<0.05$ and $P<0.01$, respectively. ND: Not done.
Fig. 4. Effect of overdoses of UK-21, FT-207 and 5-FU on sheep red blood cells-induced delayed type hypersensitivity (SRBC-DTH) in ddY mice. Mice were sensitized with $5 \times 10^5$ or $5 \times 10^7$ SRBC, s.c. into the left hind footpad and then challenged 5 days later by an s.c.-injection of $10^8$ SRBC into the right hind footpad. Drugs were given p.o. for 5 days starting the day of sensitization. The DTH intensity was evaluated by the increase of the footpad volume 24 hr after the challenge. Columns represent means±S.E. of 7 or 8 animals. *, †: Statistically significant difference from the control at $P < 0.05$ and $P < 0.01$, respectively.

Fig. 5. Effect of overdoses of UK-25, HCFU, FT-207 and 5-FU on sheep red blood cells-induced delayed type hypersensitivity (SRBC-DTH) in ddY mice. Mice were sensitized with $5 \times 10^5$ or $5 \times 10^7$ SRBC, s.c. into the left hind footpad and then challenged 5 days later by an s.c.-injection of $10^8$ SRBC in the right hind footpad. Drugs were given p.o. for 5 days starting the day of sensitization. The DTH intensity was evaluated by the increase of footpad volume 24 hr after the challenge. Columns represent means±S.E. of 8 mice. *, †: Statistically significant difference from the control at $P < 0.05$ and $P < 0.01$, respectively.
mmole/kg and 5-FU at 0.2 mmole/kg decreased the thymus weight of mice sensitized with $5 \times 10^5$ SRBC. FT-207 at 0.4 mmole/kg decreased that of mice sensitized with $5 \times 10^5$ SRBC (Table 4). Spleen weight was not much affected by any of the drugs (data not shown).

Next, the effects of the drugs at overdoses on the SRBC-DTHs were examined (Figs. 4 and 5). UK-21 showed a tendency to suppress $5 \times 10^5$ SRBC-DTH only at the highest dose of 0.8 mmole/kg and enhanced $5 \times 10^7$ SRBC-DTH at 0.4 and 0.6 mmole/kg (Fig. 4). UK-21 did not suppress $5 \times 10^7$ SRBC-DTH even at the highest dose. In contrast, FT-207 at 1.2 mmole/kg and 5-FU at each dose suppressed $5 \times 10^5$ and $5 \times 10^7$ SRBC-DTHs. FT-207 at 0.8 mmole/kg showed a tendency to enhance $5 \times 10^7$ SRBC-DTH. UK-25 at 0.6 mmole/kg enhanced both $5 \times 10^5$ and $5 \times 10^7$ SRBC-DTHs in agreement with the results of Fig. 3, and it showed a tendency to suppress $5 \times 10^5$ SRBC-DTH at the highest dose of 1.0 mmole/kg (Fig. 5). HCFU did not affect $5 \times 10^5$ SRBC-DTH but suppressed $5 \times 10^7$ SRBC-DTH significantly at 0.6 mmole/kg. The results for FT-207 and 5-FU were in close accordance with the findings in Fig. 4. All drugs decreased the thymus and spleen weights almost dose-dependently and significantly (Tables 5 and 6). The reduction of thymus weight was greater than that of spleen weight. There was not much difference between mice sensitized with $5 \times 10^5$ and $5 \times 10^7$ SRBC in the reductions of thymus and spleen weight by the drugs.

**Table 5.** Effect of overdoses of UK-21, FT-207 and 5-FU on thymus and spleen weights in mice sensitized with sheep red blood cells (SRBC)

| Sensitized with | Thymus weight | Spleen weight |
|----------------|---------------|---------------|
|               | mg (%)*        | mg (%)*        |
| UK-21 0.4     | 66±4 (100)    | 117±6 (100)   |
| 0.6           | 45±5 (68)     | 86±6 (74)     |
| 0.8           | 33±4 (49)     | 66±4 (57)     |
| FT-207 0.8    | 23±6 (35)     | 98±7 (84)     |
| 1.2           | 19±2 (29)     | 81±6 (70)     |
| 5-FU 0.4      | 20±2 (31)     | 85±5 (73)     |
| 0.6           | 12±1 (19)     | 58±2 (50)     |

See legend to Fig. 4 for details. The thymus and spleen weights were measured after the SRBC-DTH assay. Data represents the mean ± S.E. of 7 or 8 mice. *: Statistically significant difference from the control at P<0.05 and P<0.01, respectively.

**Table 6.** Effect of overdoses of UK-25, HCFU, FT-207 and 5-FU on thymus and spleen weights in mice sensitized with sheep red blood cells (SRBC)

| Sensitized with | Thymus weight | Spleen weight |
|----------------|---------------|---------------|
|               | mg (%)*        | mg (%)*        |
| Control 63±3 | 63±5 (100)    | 115±4 (100)   |
| 0.6           | 29±3 (46)     | 102±7 (81)    |
| 0.8           | 23±4 (37)     | 118±12 (93)   |
| 1.0           | 18±1 (29)     | 81±4 (64)     |
| HCFU 0.4      | 17±1 (27)     | 99±7 (78)     |
| 0.6           | 17±1 (27)     | 113±8 (89)    |
| FT-207 0.8    | 29±2 (46)     | 104±6 (82)    |
| 1.2           | 22±3 (36)     | 72±5 (57)     |
| 5-FU 0.4      | 21±1 (34)     | 104±7 (82)    |
| 0.6           | 14±1 (22)     | 68±3 (54)     |

See legend to Fig. 5 for details. The thymus and spleen weights were measured after the SRBC-DTH assay. Data represents the mean ± S.E. of 7 or 8 mice. *: Statistically significant difference from the control at P<0.05 and P<0.01, respectively.
Effect of the drugs on Meth A-DTH

The drugs were given for 10 days starting the day of tumor transplantation. Tumor size was measured, and Meth A-DTH was induced 10 and 20 days after the transplantation (Fig. 6). Tumor growth was suppressed by all the drugs dose-dependently on day 10 as well as on day 20. Meth A-DTH in the control was detected as 7.1 ± 2.2 μl on day 10, but became undetectable on day 20. On day 10, UK-21 at 0.1 and 0.2 mmole/kg and UK-25 at 0.4 and 0.6 mmole/kg enhanced the response significantly. On the other hand, FT-207 and 5-FU did not enhance the response, but rather suppressed it at the highest doses. On day 20, UK-21 at 0.2 and 0.4 mmole/kg and UK-25 at every dose enhanced the response. FT-207 and 5-FU also enhanced or tended to enhance it, although the degrees of the enhancement were much less than those of UK-21 and UK-25. Thirteen out of 16 mice given 5-FU at the dose of 0.4 mmole/kg died by day 10, and all died by day 20.

Effect of the drugs on Meth A tumor-neutralizing activity

The drugs were given for 10 days starting the day of tumor transplantation. The neutralizing activity of spleen cells of the control mice was detectable on day 10 (Figs. 7 and 8) and decreased to a level almost the same as that of normal mice (Fig. 7) or became lower than that on day 20 (Fig. 8). UK-21 tended to enhance the activity at every dose on day 10 and enhanced it significantly at 0.15 and 0.2 mmole/kg on day 20 (Fig. 7). FT-207 enhanced it significantly at 0.6 mmole/kg on day 10 and enhanced it at 0.4 mmole/kg on day 20. UK-25 tended to enhance the activity at 0.3 and 0.4 mmole/kg on day 10 and enhanced it significantly at 0.2 and 0.3 mmole/kg on day 20 (Fig. 8). HCFU showed a tendency to enhance the activity on day 10, but did not on day 20 at any dose.

DISCUSSION

5-FU is an antimetabolite used widely for many types of cancer because it shows a suppressive effect on a wide spectrum of cancers including carcinomas that are relatively resistant to chemotherapy. FT-207 is a derivative of 5-FU, which was designed to give a long lasting effective plasma concentration level of 5-FU by being metabolized gradually in the liver (18). HCFU is a derivative of 5-FU that can release 5-FU independently of liver function (19, 20). These derivatives are in clinical use and known to show lower side effects than 5-FU.

UK-21 and UK-25 did not suppress PC-DTH, but rather enhanced it, as did FT-207 and 5-FU. HCFU suppressed both PC-DTH and thymus weight. It seems that the suppressive effect of HCFU on PC-DTH and thymus weight was relatively strong. It is notable that FT-207 and
5-FU enhanced PC-DTH even at doses that reduced the thymus weight. In fact, it is well known that immunosuppressive agents including antitumor drugs show immunopotentiating action, although it depends on the experimental conditions for inducing the immune response, especially the antigen dose and timing and doses of the agents. It was possible that the enhancement of the PC-DTH response observed here was a result of an inhibition of suppressor cells involved in down-regulation of the response. The observed effect of the drugs on the immune response might be the combined result of immunostimulation and immunosuppression, making it difficult to discuss and judge their immunosuppressive potencies.

Considering these types of difficulties, further studies
to evaluate the effects of drugs on DTH were carried out by using a response to SRBC in mice, because the effects of drugs on immune responses were affected by the dose of sensitizing antigen as described above, and it was easy to control the dose for sensitization in SRBC. It is well known that SRBC-DTH in mice has an optimum sensitizing dose of SRBC. Induction of suppressor T cells has been considered as a mechanism for the reduced response by an over-sensitizing dose of SRBC. It is widely accepted that treatment with CY before antigen increases the immune response against SRBC by eliminating the precursors of suppressor T cells (21, 22), which also occurs in the case of tumor immunity (23–25). In the present results, the pretreatment with CY did not affect the response in mice sensitized with lower doses (1 × 10^5 –1 × 10^6) of SRBC and enhanced it in mice sensitized with higher doses (1 × 10^6–5 × 10^8). Therefore, effects of FT-207 and 5-FU on 5 × 10^5 SRBC-DTH and/or 5 × 10^7 SRBC-DTH were examined (Table 3). FT-207 at 0.8 mmole/kg and 5-FU at 0.4 mmole/kg suppressed 5 × 10^5 SRBC-DTH and enhanced 5 × 10^7 SRBC-DTH, while these drugs reduced the thymus weight similarly in both mice sensitized with 5 × 10^5 and 5 × 10^7 SRBC. These results suggest less participation of suppressor T cells in the response of 5 × 10^5 SRBC-DTH. Rondinone et al. (22) reported that CY-treatment of mice after sensitization with a higher dose of 1 × 10^8 SRBC enhanced the DTH response even though the response had been already augmented by CY-pretreatment. They thought the most likely target was the auxiliary cells to T suppressor cells of TDTH effectors (26). The enhancement of 5 × 10^7 SRBC-DTH by FT-207 and 5-FU given after the sensitization might occur through the same mechanism.

The effect of UK-21, UK-25 and the reference drugs on 5 × 10^5 and 5 × 10^7 SRBC-DTHs was examined in their therapeutic dose ranges as well as in the overdose ranges (see Table 7). In their therapeutic dose ranges (Fig. 3), UK-21 as well as FT-207 did not affect 5 × 10^5 SRBC-DTH, and UK-25 enhanced the response, but 5-FU at 0.2 mmole/kg suppressed it. On the other hand, all the drugs enhanced or tended to enhance 5 × 10^7 SRBC-DTH. The enhancing effects of UK-21 and UK-25 on 5 × 10^7 SRBC-DTH were remarkable. Next, the effects of the drugs on the SRBC-DTHs were examined in their overdose ranges. FT-207 and 5-FU at higher doses suppressed both 5 × 10^5 and 5 × 10^7 SRBC-DTHs, and HCFU suppressed 5 × 10^7 SRBC-DTH significantly. In contrast, UK-21 and UK-25 showed only a tendency to suppress 5 × 10^5 SRBC-DTH at the highest dose and did not suppress 5 × 10^7 SRBC-DTH at any dose. UK-25 at 0.6 mmole/kg enhanced both 5 × 10^5 SRBC-DTH and 5 × 10^7 SRBC-DTH. These results suggest that the suppressive effects of UK-21 and UK-25 on SRBC-DTH are lower than those of the other drugs. UK-25 enhanced 5 × 10^5 SRBC-DTH at the doses of 0.4 and 0.6 mmole/kg, although the other drugs including UK-21 did not enhance 5 × 10^5 SRBC-DTH at any dose. We do not yet know the mechanism for the enhancement of 5 × 10^7 SRBC-DTH by UK-25. However, because CY-pretreatment did not affect the 5 × 10^5 SRBC-DTH and none of the drugs except UK-25 enhanced it at any doses in wide ranges of their therapeutic and overdoses, it is likely that UK-25 enhanced the reaction through a mechanism other than inhibiting suppressor T cells or their auxiliary cells.

Although the suppressive effect of the drugs on the thymus weight did not necessarily have a good correlation to the suppressive effect of the drugs on the DTHs, the suppressive effect of the drugs on thymus is a serious immuno-

**Table 7.** Effect of UK-21, UK-25, 5-FUR, HCFU, FT-207 and 5-FU on thymus weight and tumor growth in mice

| Drugs | ID_{50} against thymus weight (mmole/kg) | ED_{50} against tumor growth (mmole/kg) | Therapeutic index (ID_{50}/ED_{50}) |
|-------|---------------------------------|---------------------------------|----------------------------------|
|       | 5 × 10^5 SRBC | 5 × 10^7 SRBC | Mean |       |       |
| UK-21 | 0.78  | 0.95  | 0.87  | 0.19  | 4.6   |
| UK-25 | 0.76  | 0.67  | 0.72  | 0.30  | 2.4   |
| HCFU  | —     | —     | —     | 0.15  | —     |
| FT-207| 0.72  | 0.76  | 0.74  | 0.51  | 1.5   |
| 5-FU  | 0.31  | 0.28  | 0.30  | 0.15  | 2.0   |

*4: Data from Tables 3, 4, 5 and 6 were processed by the probit method. b: Data from the preceding paper (6): BALB/c mice were transplanted with 1 × 10^6 Meth A cells, s.c. and given drugs p.o. for 10 consecutive days starting the day of transplantation. Antitumor activity was evaluated by the tumor size on day 10. Data were processed by the probit-method.*
logical side effect if the drug must be applied for a long period. Table 7 shows the doses of the drugs decreasing thymus weight by 50% (ID_{50} values) calculated from the data of the SRBC-DTH experiments. The values were statistically assessed by the probit method with a computer. Table 7 also shows the dose of the drugs suppressing the growth of Meth A tumor in BALB/c mice by 50% (ED_{50} values), which have been reported in our previous paper (6). The therapeutic indexes calculated as the ratio of the ID_{50} value to the ED_{50} value disclose that UK-21 and UK-25 have lower suppressive activity against the thymus than FT-207 and 5-FU.

Previously, we (17) reported that Meth A-DTH could be detected even in mice non-immunized but bearing the primary tumor. The Meth A-DTH was detected until 15 days after the primary transplantation, but decayed drastically by day 20 in inverse proportion to the tumor growth. Similarly, the neutralizing activity of spleen cells of BALB/c mice bearing primary Meth A tumor was detected on days 10 and 15 after the transplantation, and then it decreased on day 20 to a level lower than that of normal spleens (27). The decays of the Meth A-DTH and the neutralizing activities were also seen on day 20 in the present study. UK-21 and UK-25 enhanced Meth A-DTH on day 10 and restored the response from the decay on day 20. It seemed unlikely that the restored responses on day 20 by UK-21 and UK-25 were only a rebound effect of discontinued administration of the drugs after day 10, because these drugs did not suppress the response on day 10. On the other hand, FT-207 and 5-FU did not enhance the response but rather suppressed it at the highest doses on day 10. The extents of restoration of the response from the decay by FT-207 and 5-FU on day 20 were less than those by UK-21 and UK-25. There was not much difference among the suppressions of the tumor growth by the drugs. These results suggest that UK-21 and UK-25 have lower suppressive activity against tumor-specific DTH than FT-207 and 5-FU. North (25) reported the induction of suppressor T cells that down-regulated effector T cells in concomitant tumor immunity against Meth A tumor. However, we (17) stated previously that CY-treatment before primary Meth A tumor transplantation showed only a slight suppression of the tumor growth and little restoration from the decay of the Meth A-DTH on day 20, suggesting less participation of suppressor T cells in the decay of the immunity on day 20. We do not yet know if the restorations of the response from the decay by UK-21 and UK-25 depend on the elimination of suppressor T cells or not.

There are still much debate about which immune response of DTH effector T cells or cytotoxic T cells play a more important role in the host defense mechanisms against tumors. Both of the T cell subsets as well as the other lymphoid cells including NK cells and activated macrophages (28, 29) may co-operate. All of these cells might contribute to tumor neutralizing activity in Winn's assay. UK-21 tended to enhance the tumor neutralizing activity of spleen cells on day 10 and restored the decay of the activity on day 20. UK-25 and FT-207 also restored the activity from the decay on day 20. However, HCFU did not restore it. These results also indicate that the suppressive effects of UK-21 and UK-25 on tumor immune response are weak. Again, we presently do not know the precise mechanisms through which the drugs enhance the activity and restore it from decay on day 20.

Thus, UK-21 and UK-25 can be expected to develop as potent antitumor drugs from viewpoint of tumor immunity.

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