0014-4754/95/040343-06$1.50 + 0.20 © Birkhäuser Verlag Basel, 1995

Suppression of natural killer cell activity in mouse spleen lymphocytes by several dopamine receptor antagonists

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Received 3 May 1994; received after revision 9 September 1994; accepted 26 October 1994

Abstract. The effects of dopaminergic receptor inhibitors such as thiothixine (D_1/D_2), fluphenazine (D_1/D_2), trifluoperazine (D_1/D_2), pimozide (D_2), flupenthixol (D_1/D_2), (+/−)-SKF 83566 (D_1), and spiperone (D_2) on splenic natural killer (NK) cell cytotoxic activities were assessed in vitro using mouse spleen lymphocytes or enriched NK cells. Both the activities of the splenic NK cell cytotoxicity and the effector-target cell conjugation were suppressed by thiothixine, fluphenazine, and trifluoperazine at concentrations from 2.64 to 14.78 μM. In addition, the augmentation of the cytolytic activity of NK cells induced by interferon-α or interleukin-2 was antagonized by pretreatment with these neuroleptic compounds. However, neither the splenic NK cell cytotoxicity nor the effector-target cell conjugation were affected by treatment with other neuroleptic compounds such as pimozide, flupenthixol, (+/−)-SKF 83566, and spiperone. Thus, it appears that neuroleptic compounds such as thiothixine, fluphenazine, and trifluoperazine may act through the mechanisms other than a dopaminergic pathway to affect the NK cell–target cell interaction.
Key words. Dopamine; neuroleptics; natural killer cell; spleen lymphocytes; interferon; interleukin-2.

It has been shown that a number of neuroleptic compounds have certain effects on the immune system[1]. For example, chlorpromazine (a dopamine antagonist) reduced concanavalin A (Con A)-induced lymphocyte proliferation in vitro[2] and decreased ear swelling in a sheep erythrocyte-induced delayed type hypersensitivity in vivo[3]. Trifluoperazine, a calmodulin antagonist, reduced mitogen- and antigen-induced rabbit lymphocyte proliferation[4]. Furthermore, it was also found that chlorpromazine and trifluoperazine inhibited mitogen-induced mouse lymphocyte proliferation in vitro and in vivo[5]. On the other hand, haloperidol suppressed mouse lymphocyte proliferation in vitro and reduced picryl chloride-induced contact hypersensitivity in vivo[6]. Natural killer (NK) cells play an important role in the first line of host defense mechanisms, and they are essential for immunosurveillance against cancer and viral infections[7–10]. Although the influence of stimulation or disruption of adrenergic innervation on NK cell activity has been reported[11–15], relatively little information is available regarding the relation between dopaminergic neurons and NK cell activity in spleen lymphocytes. The present study, therefore, concentrates on the effects of several neuroleptics on NK cell activity in mouse spleen lymphocytes.

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Materials and methods

Inbred C3H/HeN mice were obtained from the Animal Center of the National Cheng Kung University (Tainan, Taiwan, R.O.C.). Male mice (7 to 10-week-old) were used in all experiments. The NK-sensitive cell line YAC-1, a Moloney virus-induced T lymphoma, was grown in stationary suspension cultures of RPMI-1640 medium (Gibco, Grand Island, NY, USA) with 8% heat-inactivated fetal bovine serum (FBS; Gibco), 2 mM L-glutamine (Sigma Chemical, St. Louis, MO, USA), 100 IU/ml penicillin G, and 100 μg/ml streptomycin. The spleens were aseptically removed and teased on a steel mesh, which was immersed in chilled RPMI-1640 in a plastic dish. The cells passed through the mesh and were washed twice with RPMI-1640. The erythrocytes were lysed with double-distilled water while the remaining cells were suspended in Dulbecco's modified Eagle's medium (DMEM, Gibco) with 10% FBS. The drugs (RBI, Natick, MA, USA) used in this study were as follows: thiothixine hydrochloride, fluphenazine hydrochloride, trifluoperazine dihydrochloride, pimozide, flupenthixol dihydrochloride, (+/−)-SKF 83566 hydrochloride, and spiperone hydrochloride. All drugs were prepared and diluted with culture medium just before use. The purified recombinant mouse interleukin-2 (IL-2, sp. act. 2.5×10^6 units/mg) was obtained from Genzyme (Cambridge, MA, USA). The mouse interferon-α (IFN-α, sp. act. 1.4×10^6 IU/mg) was purchased from Lee BioMolecular Research (San Diego,

CA, USA). Stock solutions of cytokines were prepared in culture medium and stored at −80 °C until use.

The standard 4-hour ^{51}Cr release assay was performed as described previously[15]. Briefly, the target cells were labelled with 100 μCi ^{51}Cr (Amersham International, Amersham, UK) for 1 h in 0.2 ml of DMEM containing 10% FBS. After washing three times in RPMI-1640, effector cells at various concentrations were incubated with 1×10^4 ^{51}Cr-labelled target cells in 96 well round-bottom microplates (Corning Laboratory Sciences, Corning, NY, USA). Effector-to-target cell ratios of 160:1, 80:1, or 40:1 were made by different dilutions of 50 μl of effector cells, 50 μl of target cells and 100 μl of culture medium or tested drugs in each well. After incubation, the plates were centrifuged at $800 \times g$ for 10 min, and the radioactivity of the supernatant in 0.1 ml was measured in a gamma counter (Compu-Gamma 1282, LKB, Sweden). The percentage of specific lysis was calculated by the following formula:

$$\% \text{ specific cytolysis} = \frac{\text{test cpm-spontaneous cpm}}{\text{maximal cpm-spontaneous cpm}} \times 100$$

Test cpm = cpm released in the presence of effector cells; spontaneous cpm = cpm released from target cells added to culture medium alone; and maximal cpm = cpm released from target cells obtained by adding 0.2% sodium dodecyl sulfate (Merck, Darmstadt, Germany). Each treatment was tested in triplicate. Spontaneous release was less than 12% of the maximal release. The natural killer (NK) cell fractions of nylon wool nonadherent spleen lymphocytes were enriched by Percoll (Pharmacia LKB Biotechnology, Uppsala, Sweden) discontinuous-density gradient purification as previously described[16,17]. Briefly, 8.964 ml of 10 × phosphate buffer (PBS, magnesium, calcium free) was diluted with 90 ml Percoll, 0.46 ml of 1 N HCl and 1.0 ml Hepes buffer (Gibco) to produce a 90% Percoll solution (density 1.12 g/ml). Seven different concentrations of Percoll in PBS were prepared, ranging from 38.6 to 70.1% with 4.5% concentration steps. The top fraction, fraction 0 (F.0), was 38.6% Percoll; the next was 47.6% (F.1), and the last fraction (F.6) was 70.1%. After a careful layering of the gradients into 15 ml conical test tubes (Corning Laboratory Sciences, Corning, NY, USA), 5 to 20×10^7 nylon wool nonadherent

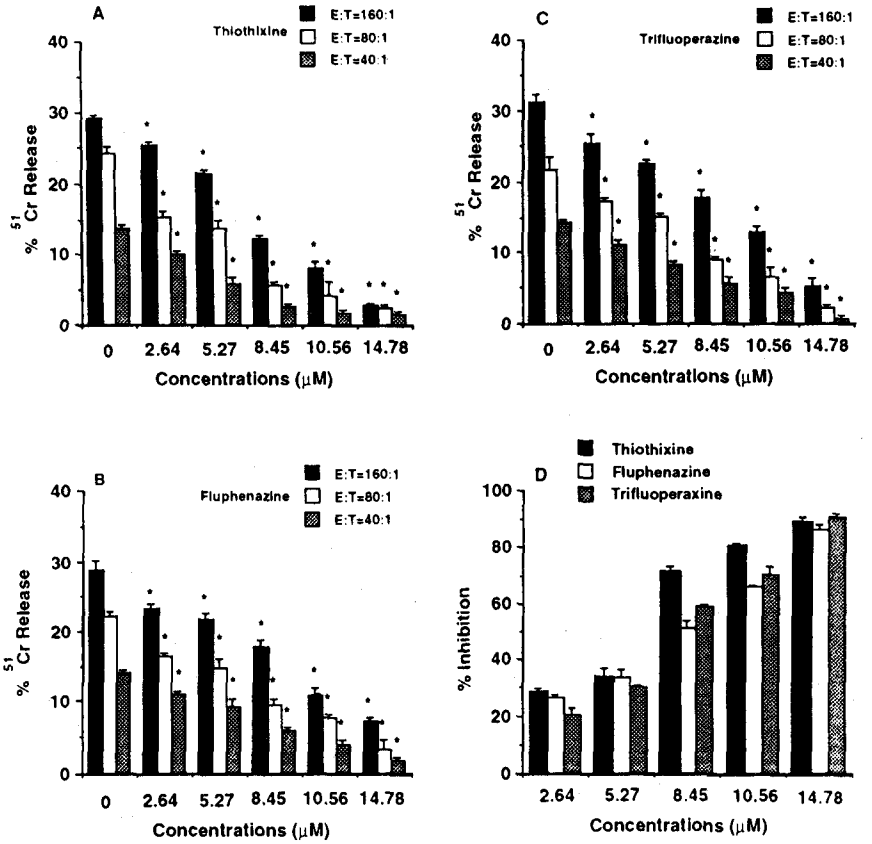

Figure 1. Dose-dependent inhibition by thiothixine, fluphenazine and trifluoperazine on mouse splenic NK cell activity. Thiothixine, fluphenazine and trifluoperazine were used at various concentrations for the splenic NK cytotoxicity assay. Cell cytotoxic activity against YAC-1 cells at effector to target ratios of 160:1, 80:1, or 40:1 was assessed in a 4-h ^{51}Cr-release assay. *A* Thiothixene, *B* Fluphenazine, *C* Trifluoperazine. *D* The calculation of percentage of inhibition was described in 'Materials and methods'. The values are expressed as means ± SEM of three experiments. Each experiment used four mice. * $p < 0.05$ compared to medium control group.

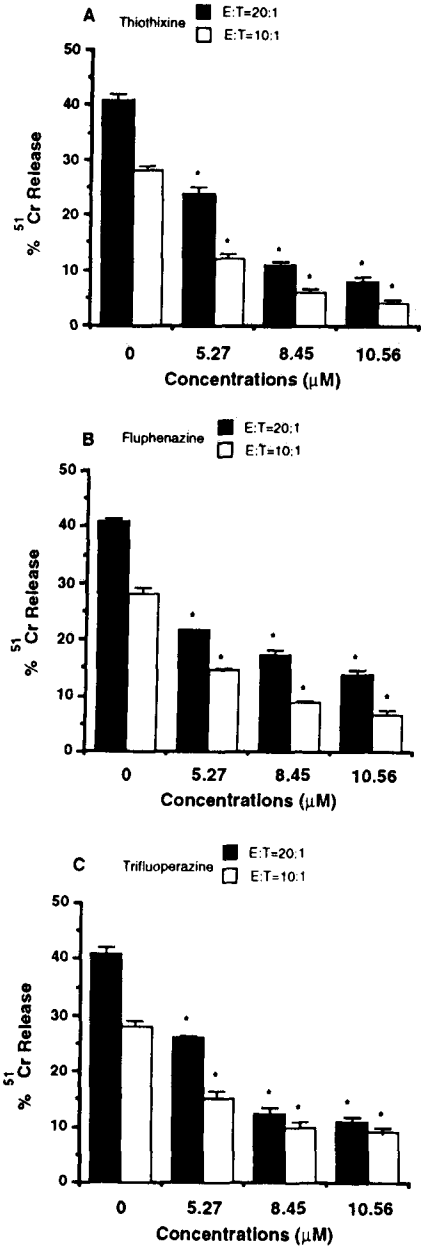

Figure 2. Dose-dependent inhibition by thiothixine, fluphenazine and trifluoperazine on the activity of enriched mouse NK cells. Thiothixine, fluphenazine and trifluoperazine were used at various concentrations for NK cytotoxicity assay. Plastic nonadherent cells were filtered over nylon wool, and nonadherent cells were fractionated over Percoll gradients. Cells from the low density Percoll fraction were assessed. Cell cytotoxic activity against YAC-1 cells at effector to target ratios of 20:1, or 10:1 was assessed in a 4-h ^{51}Cr-release assay. *A* Thiothixene, *B* Fluphenazine, *C* Trifluoperazine. The values are expressed as means ± SEM of three experiments. Each experiment used four mice. * $p < 0.05$ compared to medium control group.

spleen lymphocytes were placed on the top of the gradient and the tube was centrifuged at 300 × *g* for 30 min at room temperature. The F.1 fractions were collected with a Pasteur pipette from the top and were washed twice with PBS. Giemsa-stained cytocentrifuge cell smears were examined by cell morphology and a 85–90% NK cell population was found in F.1. Effector-target cell conjugate formation was performed as described previously[15,18]. Briefly, equal numbers (usually 2.5×10^5) of cells from suspensions of the effector and target cells in RPMI 1640 + 10% FBS were mixed in Eppendorf polypropylene centrifuge tubes (1.5 ml) to a total volume of 0.5 ml. The tubes were incubated for 10 min at 37 °C. After incubation, the cells were pelleted by centrifugation at 600 × *g* for 5 min. The supernatant was aspirated, leaving approximately 0.2 ml in which the pellet was suspended by 10 times trituration with a Pasteur pipette. The effector-target cell conjugation was counted using a hemocytometer. The percentage of conjugating lymphocytes was calculated by counting the number of single lymphocytes bound to single target cells per 400 effector cells. Statistical significance was determined by the paired Student's *t*-test.

Results and discussion

Figure 1 shows the mean values of the NK cell activity from spleen lymphocytes in medium with or without drug treatment. The splenic NK cytotoxic activity against YAC-1 target cells was measured by the 4 h ^{51}Cr-release assay after test-agents were added. The result shows that thiothixine, fluphenazine or trifluoperazine, at concentrations from 2.64 to 14.78 μM, suppressed the splenic NK cytotoxic activity in a dose-related manner (fig. 1A, 1B and 1C). However, other neuroleptic compounds such as pimozide, fluspenthixol, (+/−)-SKF 83566, and spiperone, at concentrations from 2.64 to 14.78 μM, were found to have no effect on the splenic NK cytotoxic activity (data not shown here). The inhibitory effect of thiothixine, fluphenazine or trifluoperazine on the splenic NK cytotoxic activity was observed at all effector-target cell ratios tested and was greatest within the dose range 8.45–14.78 μM (fig. 1D). When these drug concentrations were increased to 14.78 μM, the splenic cytotoxic activity was completely suppressed. No suppression was observed at concentrations less than 2.64 μM in our experiments. These results were reproducible. The viability of the splenocytes was not affected by the drugs at these concentrations as assessed by trypan blue exclusion. Furthermore, figure 2 shows that thiothixine, fluphenazine or trifluoperazine, at concentrations from 5.27 to 10.56 μM, also suppressed the cytotoxic activity of enriched splenic NK cells in a dose-dependent fashion. In addition, pretreatment of the effector and target cells with 10.56 μM of the drugs for 3 h at 37 °C, followed by washing with RPMI-1640, did not suppress the cytotoxic activity of the splenic NK cell by the 4 h cytotoxic assay (fig. 3A and 3B).

The effect of thiothixine, fluphenazine or trifluoperazine on the affinity of effector cells to YAC-1 target cells was evaluated by the effector-target cell conjugation binding

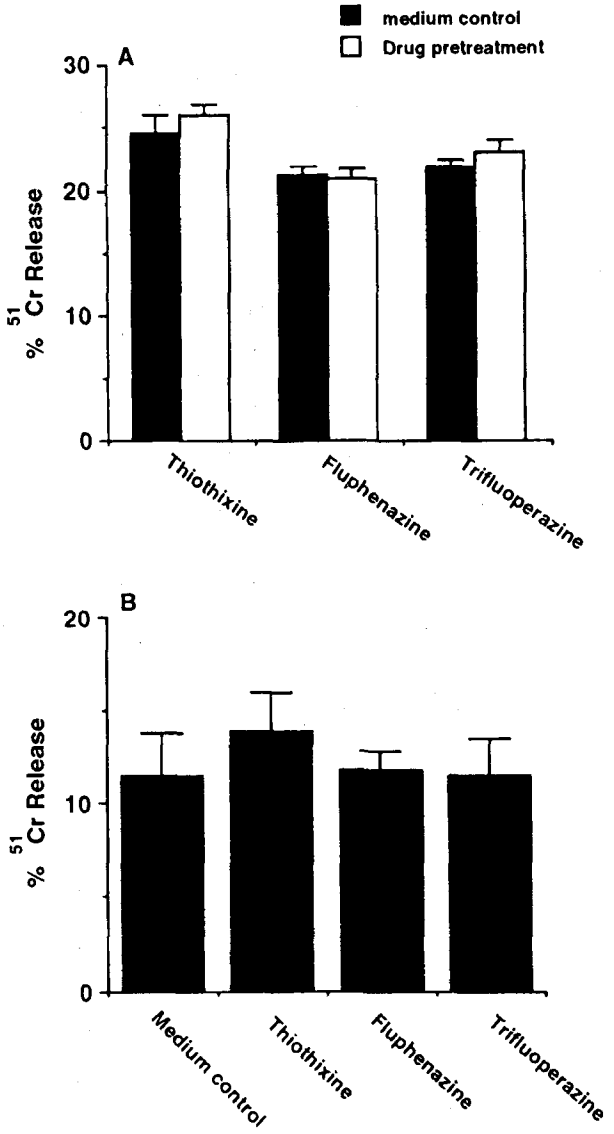

Figure 3. Inhibition of thiothixine, fluphenazine or trifluoperazine on target and effector cells. *A* YAC-1 target cells (5×10^5 cells/ml) or *B* the effector cells (2×10^7 cells/ml) were pretreated with 10.56 μM of indicated drugs for 3 h, and then assayed for cell cytotoxic activity using the 4-h ^{51}Cr release assay at the effector to target ratio of 80:1. Data shown are expressed as the means ± SEM of three experiments. Each experiment used three mice.

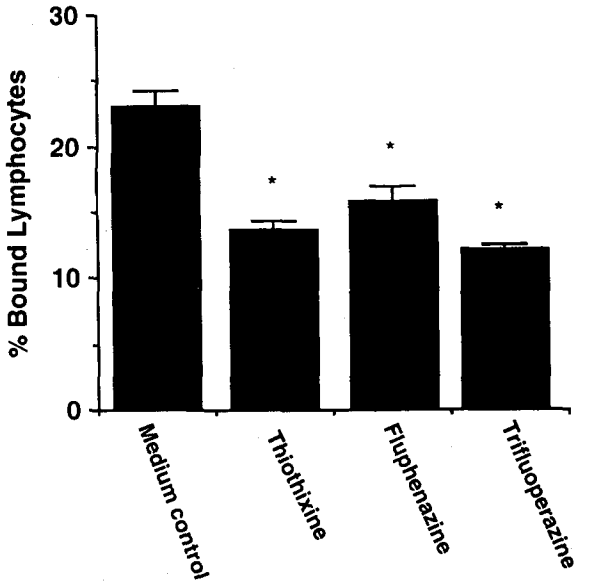

Figure 4. Conjugation of effector to target cells after thiothixine, fluphenazine or trifluoperazine treatment. Effector and target cells were mixed in a 1:1 ratio (2.5×10^5 cells) in Eppendorf polypropylene centrifuge tubes (1.5 ml) in a total volume of 0.5 ml. The tubes were incubated for 10 min at 37 °C. After incubation, the cells were pelleted by centrifugation at $600 \times g$ for 5 min. The supernatant was aspirated, leaving approximately 0.2 ml in which the pellet was suspended by 10 times trituration with a Pasteur pipette. The effector-target cell conjugation was counted using a hemocytometer. The percentage of conjugating lymphocytes was calculated by counting the number of single lymphocytes bound to single target cells per 400 effector cells. Data shown are expressed as the means ± SEM of three experiments. Each experiment used three mice. * $p < 0.05$ compared to medium control group.

assay. Significant inhibition of splenic NK cell binding activity was observed in the presence of 10.56 μM of thiothixine, fluphenazine or trifluoperazine (fig. 4).

To reverse the inhibition by thiothixine, fluphenazine or trifluoperazine of the splenic NK cell activity, interferon-α (IFN-α) or interleukin-2 (IL-2) was applied. The effector cells were pretreated with IFN-α (1000 IU/ml) or IL-2 (100 IU/ml) or IFN-α + IL-2 for 18 h, washed and then added to the target cells for a 4 h co-culture in the absence or presence of various concentrations of thiothixine, fluphenazine or trifluoperazine. Figure 5 (A, B and C) shows that the inhibitory effect of thiothixine, fluphenazine or trifluoperazine was reversed after IFN-α or IL-2 pretreatment, when the concentration was at 10.56 μM or less, but not at 14.78 μM or higher. In addition, figure 5B shows that IFN-α blocked the inhibition of fluphenazine at a concentration of 14.78 μM or less, but failed to do so at concentrations higher than 19 μM (data not shown). However, the combination of IFN-α and IL-2 completely reversed the inhibition caused by thiothixine, fluphenazine or trifluoperazine even at a concentration of 14.78 μM (fig. 5A, 5B and 5C).

Our results show that thiothixine (D_1/D_2 receptor antagonist), fluphenazine (D_1/D_2 receptor antagonist), and trifluoperazine (D_1/D_2 receptor antagonist) significantly suppress the splenic NK cytotoxicity in a dose-dependent fashion in vitro, and that the inhibition was not caused by the toxicity of those drugs to the mouse splenic lymphocytes or target cells, as demonstrated by the ^{51}Cr-release assay or trypan blue exclusion. These dopaminergic receptor antagonists may act through the inhibition of effector-target cell conjugation to reduce NK cell cytolytic activity.

Both in vitro and in vivo data show that interferons and interleukin-2 can enhance the NK cell growth and cyto-

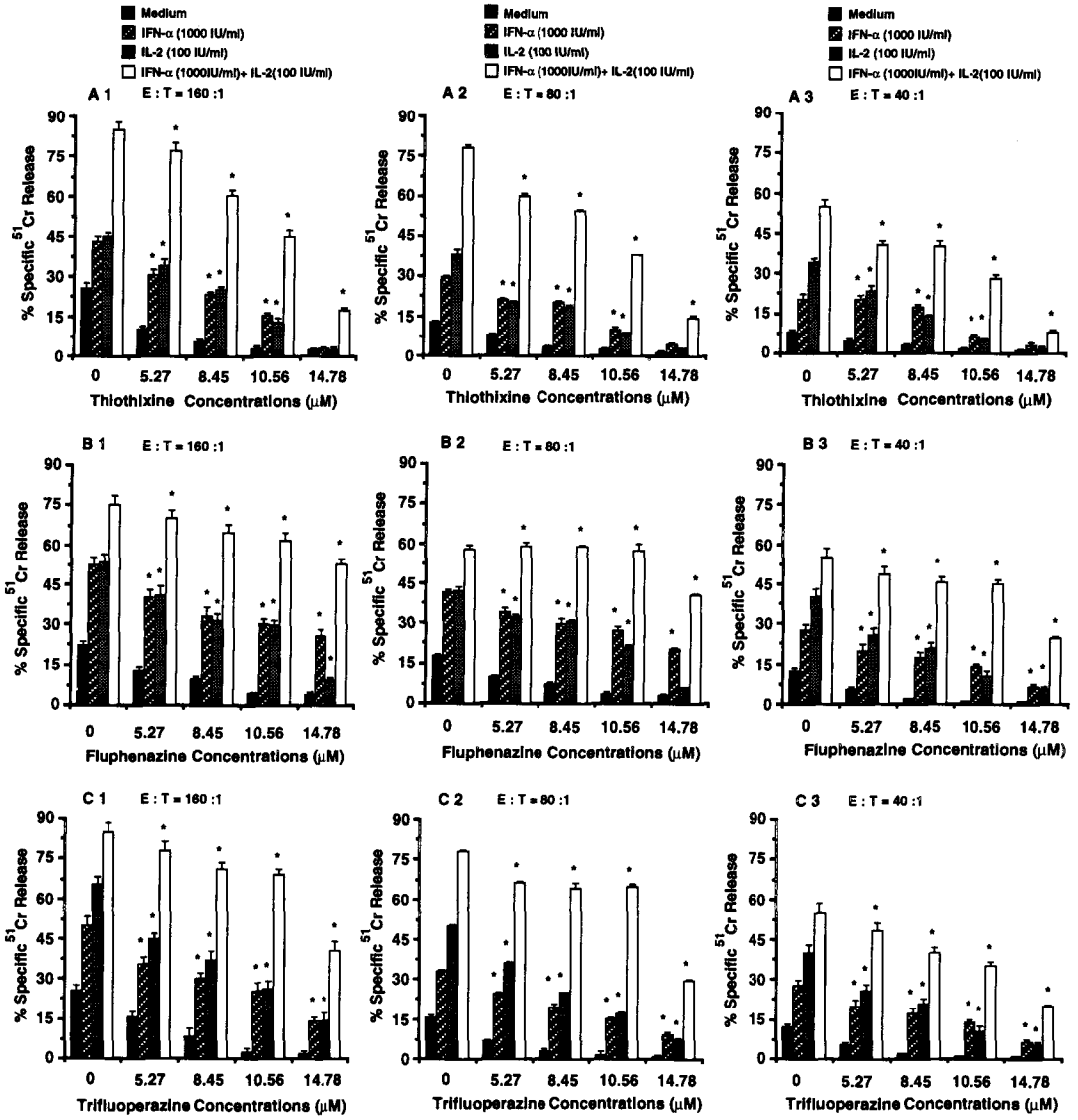

Figure 5. Inhibitory effects of thiothixine, fluphenazine or trifluoperazine on cells after IFN-α, rIL-2, or IFN-α + rIL-2 pretreatment. The effector cells were pretreated with either IFN-α (1000 IU/ml), rIL-2 (200 IU/ml) or IFN-α (1000 IU/ml) + rIL-2 (200 IU/ml) for 18 h, and washed three times with DMEM containing with 10% FBS. Thiothixine (A1, A2, and A3), fluphenazine (B1, B2, and B3) or trifluoperazine (C1, C2, and C3) were present at concentrations indicated throughout the NK cytotoxicity assay. Cell cytotoxic activity against YAC-1 cells at effector to target ratios of 160:1, 80:1, or 40:1 were assessed at a 4-h ^{51}Cr-release assay. The values are expressed as the means ± SEM of three experiments. Each experiment used three mice. (A1, B1, C1) E:T = 160:1; (A2, B2, C2) E:T = 80:1; (A3, B3, C3) E:T = 40:1. * $p < 0.05$ compared to medium control group.

toxic activity in man and animals[19–23]. The present results also demonstrate that the augmentation of NK cell cytolytic activity induced either by IFN or IL-2 is antagonized by these neuroleptic compounds (such as thiothixine, fluphenazine, and trifluoperazine).

The enhancement of NK cytolytic activity after 4 to 6 h of IFN exposure requires RNA synthesis and is transient[24–26]. IL-2 induces increased expression of some NK cell surface adhesion molecules, apparently by mediating the induction of cytotoxicity for NK-resistant targets[27]. An increase in number and size of NK cell cytolytic granules has also been demonstrated following IL-2 activation[28]. Furthermore, IL-2 stimulates NK cell expression of mRNA for some serine proteases[29]. Since all of these changes require several days of IL-2 exposure, they cannot explain the earliest increase in NK cytotoxic activity and the influence exerted by neuroleptic compounds. The neuroleptic drugs such as thiothixine, fluphenazine or trifluoperazine could suppress the NK cell cytolytic activity in vitro, but other compounds possessing neuroleptic activity – namely pimozide (D_2 receptor antagonist), flupenthixol (D_1/D_2 receptor antagonist), (+/−)-SKF 83566 (D_1 receptor antagonist), and spiperone (D_2 receptor antagonist) – had no effect on either the splenic NK cytotoxicity or the target-effector cell conjugation. In fact, several dopaminergic an-

tagonists (e.g. sulpiride, metroclopramide and haloperidol) do not share the same immunological profiles as chlorpromazine and trifluoperazine[5]. Nevertheless, we find it prudent not to conclude that a dopaminergic pathway is involved in NK cell-target cell interaction. It could well be that these inhibitors act by pathways which are still unknown.

Acknowledgements. The work reported here was supported by a grant from National Science Council (Taipei, Taiwan, Republic of China: NSC 82-0412-B-006-M8).

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