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SHORT COMMUNICATION

ACTIVATION OF SPLEEN CELL LYTIC ACTIVITY BY THE ALKALOID THIOPHOSPHORIC ACID DERIVATIVE: UKRAIN

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Abstract — Ukrain is a semisynthetic compound consisting of alkaloids from *Chelidonium majus* L. conjugated to thiophosphoric acid, with immunomodulatory and therapeutic properties in cancer patients. The present *in vitro* studies demonstrate that Ukrain is an effective biological response modifier in that it augmented, by up to 48-fold, the lytic activity of spleen lymphocytes obtained from alloimmunized mice. The lytic activities of IL-2-treated spleen cells and peritoneal exudate lymphocytes were also increased significantly by the addition of Ukrain to the CML assay medium. The highest Ukrain-induced enhancement of spleen lymphocyte lytic activity *in vitro* was found to occur at 18 days after alloimmunization, was dose dependent and specific for the immunizing P815 tumor cells. Since Ukrain was present only during the CML assays, its mode of action is thought to be via direct activation of the effector cell's lytic mechanism(s).

The discovery and development of immune response modifying agents, which could be effective in a clinical setting for the treatment of cancer, has become a promising and important goal in immunotherapy. The use of various biological response modifiers (BRMs) which enhance the host immune response, with or without the *in vitro* activation of immune effector cells, is an active area of investigation (Herberman, 1985; Rosenberg & Lotze, 1986). The full therapeutic potential of the various BRMs has not been fully realized due to their undesirable side-effects when used at the concentrations required for maximum biological activity (Lotze, Frana, Sharrow, Robb & Rosenberg, 1985a; Lotze *et al.*, 1985b; Urba & Longo, 1986). Thus, the identification and characterization of new compounds without deleterious side-effects would enhance the prospects of immunotherapy as a practical and effective cancer treatment modality.

A new and promising area of research has evolved from biochemical phytotherapy, where plant alkaloids as well as their semisynthetic derivatives have been found to be effective immunomodulators in animal models (Dennis, 1986; Humphries, Matsumoto, White, Molyneux & Olden, 1988; Mohla *et al.*, 1990) and in cancer patients (Nowicky, Staniszewski, Zbroja-Sontag, Slezak, Nowicky & Hiesmayr, 1991). For example, the indolizidine alkaloid swainsonine, has been reported to inhibit experimental lung metastasis of B16-F10 melanoma cells when administered systemically to C57BL/6 mice in a prophylactic setting. The inhibition of pulmonary metastasis was found to be mediated by the host's NK cells. Moreover, swainsonine induced an increase in murine splenocytes and macrophage cytotoxicity, proliferation of bone marrow cells as well as HLA class I expression on human breast carcinoma cells (Humphries *et al.*, 1988; Mohla *et al.*, 1990).

Similarly, alkaloids from the plant *Chelidonium majus* L. (Han, Nowicky & Gutman, 1991) conjugated to thiophosphoric acid, yield a triaziridide compound denoted as Ukrain which, in preliminary clinical studies, has been found to have immunomodulatory activity (Nowicky *et al.*, 1991; Nowicky, Liepins, Zbroja-Sontag, Staniszewski & Danilos, 1990). The major immune parameters affected by this alkaloid derivative consist of an increase in the

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total T-lymphocyte number and a normalization of the T-helper/T-suppressor cell ratios, without affecting serum immunoglobulin levels, complement components and acute phase proteins (Nowicky *et al.*, 1991).

The present studies were undertaken to investigate *in vitro* the possible mechanism by which the alkaloid derivative Ukrain, may mediate its therapeutic effects in cancer patients (Nowicky *et al.*, 1991). The results of the present *in vitro* studies demonstrate that Ukrain increased significantly the lytic activity of spleen lymphocytes obtained from alloimmunized mice. The enhanced lytic activity was dose dependent and directed against the immunizing H-2^d P815 mastocytoma tumor cells. Furthermore, the time course after alloimmunization at which maximal *in vitro* enhancement of spleen lymphocyte lytic activity could be obtained was found to occur at 18 days, at which time a 48-fold increase of lytic activity could be demonstrated by the addition of Ukrain to the CML assay medium. Lesser, but nevertheless significant, Ukrain-induced increase in spleen lymphocyte lytic activity could also be demonstrated at days 6, 12, 26 and 34 after *in vivo* alloimmunization. The lytic activity of peritoneal exudate lymphocytes could also be augmented by the addition of Ukrain to the CML assay medium. However, in all instances, spleen lymphocytes which had no significant endogenous lytic activity displayed the greatest increase by the addition of Ukrain to the CML assay medium.

EXPERIMENTAL PROCEDURES

Tumor cells. Mastocytoma P815 and the AKR leukemia AKIL cell lines (ATCC, Rockville, MD) were maintained in DMEM medium supplemented with 8.0% bovine fetal calf serum (GIBCO Labs, Grand Island, NY) and containing penicillin and streptomycin, 100 units/ml and 100 μg/ml, respectively. Both cell lines were cultured at 37°C in a humidified atmosphere of 5% CO_2 – 95% air.

Effector cells. C57BL/6 mice (H – 2^b) were immunized with a single intraperitoneal injection of 1×10^6 P815 mastocytoma (H – 2^d) cells. At various times after immunization spleen and peritoneal exudate cells were harvested and used as effector cells. Peritoneal lymphocytes were obtained by peritoneal lavages with 5.0 ml of DMEM medium containing 10 units/ml of heparin. Peritoneal exudate cells were washed in DMEM medium to remove heparin, transferred to 75 cm^2 tissue culture flasks and incubated at 37°C for 2 h to remove adherent cells. Spleen lymphocytes were obtained by mincing the spleens with a scalpel followed by pipeting the fragments against the bottom of a Petri dish to disrupt follicles. Red blood cells were lysed by 0.1 M Tris – ammonium chloride followed by three washes in DMEM – 8% FCS medium. Spleen plastic adherent cells were removed by the same procedure used for peritoneal exudate cells, as described above. Both, peritoneal exudate and spleen non-adherent cell viability was determined by trypan blue exclusion before using them as effector cells in cell-mediated lysis assays. Both peritoneal exudate and spleen cells were used as effector cells without secondary *in vitro* stimulation.

Cell mediated-lysis (CML) assays. Mastocytoma P815 and the AKIL and EL-4 leukemia cells were labelled with 200 μCi of Na_2 $^{51}CrO_4$ (New England Nuclear Corp., Boston, MA) for 1 h at 37°C, washed in DMEM – FCS and used as targets in CML assays. CML assays were carried out in 96-well v-bottom plates containing 1×10^4 ^{51}Cr-labelled target tumor cells and 5×10^4 effector cells per well, for an E : T ratio of 5 : 1. Multiwell plates were centrifuged at 1000 rev/min/5 min and incubated at 37°C for 3.5 h in a humidified 95% air and 5% CO_2 atmosphere. After this incubation period the specific ^{51}Cr release was determined by harvesting 150 μl of supernatant per well and radioactivity counts measured in a Packard gamma-counter. The percentage of specific ^{51}Cr release was calculated according to the following formula:

$$\%\ \text{Specific}\ ^{51}\text{Cr release} = \frac{(\text{effectors} + \text{targets} + \text{drug}) - (\text{spontaneous release} + \text{drug})}{(\text{total}\ ^{51}\text{Cr releasable}) - (\text{spontaneous release} \pm \text{drug})} \times 100.$$

Effects of Ukrain on effector cell-mediated lysis of tumor cells. The possible modulation of effector cell's lytic activity by the alkaloid thiophosphoric acid derivative Ukrain, was investigated in *in vitro* CML assays. The molecular weight of this alkaloid thiophosphoric acid conjugate, i.e. Ukrain, is 1470 and is freely soluble in water or tissue culture medium. Serial dilutions of Ukrain were prepared in PBS buffer to yield final concentrations ranging from 37.8 down to 0.59 μM after the addition of target and effector cells suspended in DMEM – FCS culture medium. The effects of Ukrain on the spontaneous ^{51}Cr release by the target tumor cells

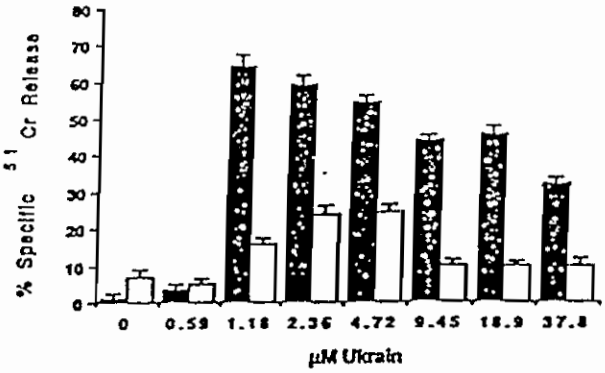

Fig. 1. Effects of the alkaloid derivative Ukrain on the cytolytic activity of spleen (■) and peritoneal (□) lymphocytes from alloimmunized mice. The lytic activity of both effector cell populations were increased in a dose-dependent manner by the presence of Ukrain in the CML assay medium. Spleen lymphocyte lytic activity was increased from 1.35 ± 1.0%, in the absence of drug, to 65.0 ± 2.5% in the presence of 1.18 μM of Ukrain, i.e. a 48-fold increase in specific lysis. The peritoneal lymphocyte lytic activity was increased from 7.0 ± 2%, in the absence of drug, to 25.0 ± 2.3% in the presence of 2.36 or 4.72 μM of Ukrain, i.e. a 3.75-fold increase in specific lysis (CML assays: E : T = 5 : 1; 3.5 h).

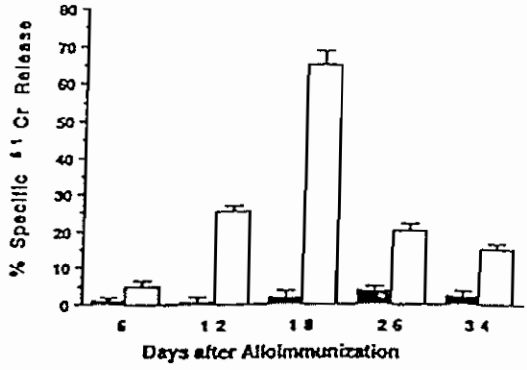

Fig. 2. Effects of 1.18 μM of Ukrain on the cytolytic activity of spleen cells harvested at various time intervals after alloimmunization. Spleen cells were harvested at days 6, 12, 18, 26, 34, and assayed for cytolytic activity in the absence (■) and presence of 1.18 μM of Ukrain in the CML assay medium (□). The highest Ukrain-induced increase of spleen cell lytic activity was found to occur at day 18 after alloimmunization. The Ukrain-induced increase in spleen cell lytic activity was significant ($P \leq 0.05$) at all post-alloimmunization times investigated (CML assays: E : T = 5 : 1; 3.5 h).

was also determined and found to be not higher than that of control cells. The percentage of specific ^{51}Cr release was calculated as described in the preceding section.

RESULTS

Dose-dependent activation of spleen and peritoneal exudate lymphocyte lytic activity by Ukrain. Preliminary experiments were carried out to determine the concentration range within which the alkaloid derivative Ukrain had an effect on the lytic activity of lymphoid effector cells without affecting the spontaneous ^{51}Cr release of tumor target cells. The concentration range of Ukrain found to fulfil these criteria was 0.6–38.0 μM.

When freshly isolated spleen lymphocytes from alloimmunized C57Bl/6 mice were used as effector cells, without secondary *in vitro* activation, they were found to have no significant endogenous lytic activity. However, the addition of Ukrain to the CML assay medium produced a significant and dose-dependent activation of spleen cell lytic activity (Fig. 1). In the presence of 1.18 μM of Ukrain, the lytic activity of spleen lymphocytes increased from 1.35 to 65.0% specific lysis, i.e. a 48-fold increase. Similarly, peritoneal exudate lymphocytes (PEL) harvested from the same animals has an endogenous

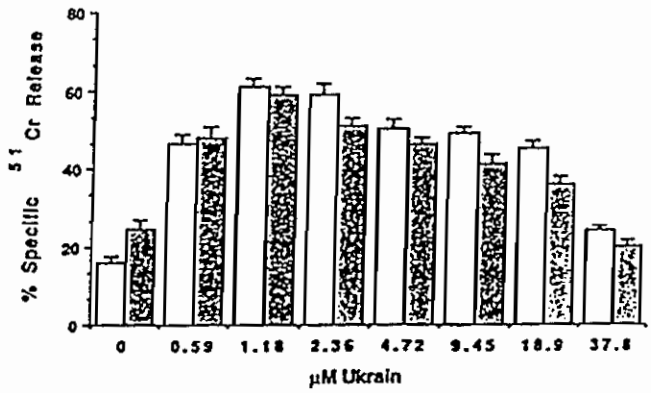

Fig. 3. Effects of Ukrain on *in vivo* primed (14 days) spleen cells followed by 4 days *in vitro* culture, without and with 10 units/ml rIL-2. The lytic activity of spleen cells cultured *in vitro* without rIL-2 was 16.3 ± 1.5% (□), which in the presence of 1.8 μM of Ukrain was further increased to 61.2 ± 2.0%, i.e. a 3.75-fold increase in specific lysis. The lytic activity of spleen cells cultured *in vitro* with 10 units/ml of rIL-2 (replenished after 48 h) was 25.0 ± 2% (□), which in the presence of 1.18 μM of Ukrain was increased to 59.0 ± 1.9%, i.e. 2.36-fold increase in specific lysis (CML assays: E : T = 5 : 1; 3.5 h).

lytic activity of 7.0%, which in the presence of 2.36 or 4.72 μM of Ukrain was increased to 25% specific lysis (Fig. 1). It should be noted that the Ukrain-induced enhancement of lytic activity remained target specific in that only mastocytoma P815

($H-2^d$) were lysed, whereas the non-specific AKR ($H-2^k$) and EL-4 ($H-2^b$) leukemia cells were not, by either effector cell source. All CML assays were carried out simultaneously in triplicate and at the low effector to target cell ratio of 5 : 1 and for 3.5 h.

Time course after alloimmunization and Ukrain inducible activation of spleen cell lytic activity. Having determined the optimal concentration of Ukrain which induced maximal activation of primed spleen cells lytic activity, i.e. 1.18 μM (Fig. 1), its effects on spleen cells harvested at various time intervals after alloimmunization were investigated. For this purpose, spleen cells were obtained from C57Bl/6 mice at 6, 12, 18, 26 and 34 days after alloimmunization with P815 mastocytoma cells and assayed for lytic activity in the presence and absence of 1.18 μM of Ukrain in the CML assay medium. As illustrated in Fig. 2, Ukrain-induced activation of spleen cell lytic activity could be detected as early as 6 days after alloimmunization, followed by a further increase at day 12, and with the maximal enhancement of lytic activity occurring at day 18. After day 18, the Ukrain-dependent activation of spleen cell lytic activity declined in a time-dependent manner. The Ukrain-induced activation of spleen cell's lytic activity was statistically significant ($P<0.05$) with respect to controls, at all time intervals after alloimmunization.

Effect of Ukrain on the lytic activity of in vivo *primed spleen cells followed by* in vitro *culture with or without rIL-2.* To investigate whether Ukrain could enhance the lytic activity of IL-2-treated cells, spleen cells harvested 14 days after *in vivo* alloimmunization, were cultured *in vitro* for 4 days in the presence and absence of 10 units/ml of rIL-2. The lytic activity of spleen cells cultured *in vitro* without IL-2 was 16.3%, whereas that of cells cultured in the presence of IL-2 was 25.0% specific lysis (Fig. 3). The addition of Ukrain to the CML assay medium, increased the lytic activity of both effector cell populations in a dose-dependent manner. Interestingly, Ukrain at the low concentration of 0.59 μM, induced a significant increase in lytic activity of these effector cells. A further increase in lytic activity occurred at 1.18 and 2.36 μM of Ukrain, followed by a gradual decline at higher concentrations (Fig. 3). It is worth noting that the Ukrain-induced increase in lytic activity of both effector cell populations reached its maximum at 1.18 μM, i.e. 61.2 and 59.0%, respectively. This increase in lytic activity represents a 3.7 and 2.4-fold increase, for spleen cells cultured in DMEM and in DMEM + 10 units/ml of IL-2, respectively. Thus, the *in vitro* culture of *in vivo* primed spleen cells produced an increase in their lytic activity which was further enhanced by the presence of Ukrain in the CML assay medium (Fig. 3).

DISCUSSION

Certain plant alkaloids have been found to favorably modulate the host's immune response to tumors in terms of reduced metastasis (Dennis, 1986; Humphries *et al.*, 1988), increased splenocyte and macrophage cytotoxicity as well as inducing an increase in HLA Class I antigen expression on human breast carcinoma cells (Mohla *et al.*, 1990). The immunomodulatory effects so far reported, occurred in a prophylactic setting, by pretreatment of animals and/or tumor cells with the alkaloids under investigation (Humphries *et al.*, 1988; Newton *et al.*, 1989). On the other hand, the alkaloid thiophosphoric acid triaziridide, Ukrain, has been found to modulate cellular immune parameters in cancer patients (Nowicky *et al.*, 1991). The major immune parameters affected by Ukrain were found to consist of an increase in patient's total T-lymphocyte and NK cell numbers, normalization of T-helper/T-suppressor cell ratios, without affecting serum immunoglobulin, complement and acute phase proteins.

Our present studies were undertaken in order to determine whether Ukrain would have a direct *in vitro* effect on the lytic activity of *in vivo* primed spleen lymphocytes. The results of our studies demonstrate that this compound had a direct and dose-dependent effect on the cytolytic activity of spleen lymphocytes from alloimmunized mice when assayed *in vitro*. A 48-fold increase in spleen lymphocyte lytic activity could be obtained by the presence of 1.18 μM of Ukrain in the CML assay medium. Furthermore, the Ukrain-induced activation of spleen cell lytic activity was specific for the mastocytoma P815 tumor cells used for alloimmunization, insofar that AKR ($H-2^k$) and EL-4 ($H-2^b$) leukemia cells, used as control target cells in all experiments were not lysed. It is significant to note that *in vivo* primed spleen cells harvested 18 days after alloimmunization had no significant endogenous lytic activity, i.e. 1.3% specific lysis, whereas in the presence of Ukrain their lytic activity increased to 65.0% (cf. Fig. 1). On the other hand, peritoneal exudate lymphocytes harvested from the same animals had an endogenous lytic activity of 7.0%, and could be increased to 24–25% specific lysis by the addition of 2.3 and 4.7 μM of Ukrain to the

CML assay medium. Thus, peritoneal exudate cells were far less susceptible than spleen cells with respect to Ukrain-mediated activation of lytic activity. The reasons for these major differences in the Ukrain-induced activation of specific lysis by these two effector cell populations are not understood at the present time.

Since most immune effector mechanisms to antigens are not constant, we investigated the Ukrain inducible activation of spleen cell lytic activity at various time intervals after alloimmunization. A significant Ukrain-induced enhancement of spleen cell lytic activity could be detected as early as 6 days after alloimmunization, with a further increase at 12 days and maximal activation occurring at 18 days, following a decrease at 26 and 34 days, cf. Fig. 2. These results indicate, albeit indirectly, that Ukrain exerts its effects on the spleen lymphocytes used as effector cells and not on the tumor target cells by making the latter more susceptible to lysis. The latter interpretation of our results is further substantiated by the finding that peritoneal exudate lymphocytes, harvested at 12–14 days after alloimmunization, which had an endogenous lytic activity of 70.0% specific lysis in our standardized CML assays (E : T = 5 : 1; 3.5 h), could not be further increased by addition of Ukrain to the CML assay medium (data not shown).

Other alkaloids, such as swainsonine, when systematically administered to nude mice in conjunction with estradiol 17-β, before implantation of subcutaneous MCF-7 cell xenografts, was found to increase the spleen cell lytic activity against YAC targets. However, no direct *in vitro* activation of spleen cell lytic activity was reported in those studies (Mohla *et al.*, 1990). Interestingly, the lytic activity of macrophages obtained from thioglycolate-treated mice and preincubated *in vitro* with swainsonine, were found to have an enhanced lytic activity against the human breast carcinoma cell line MDA-MB-231. *In vivo* pretreatment of mice with swainsonine were reported to result in a greater enhancement of macrophage lytic activity (Mohla *et al.*, 1990). Since *in vitro* activation of antigen specific effector cell lytic activity may be of clinical relevance, we investigated the effects of Ukrain on *in vivo* primed spleen lymphocytes followed by *in vitro* culture with low concentration of IL-2. Our results showed that the lytic activity of these cells could be further enhanced by the addition of Ukrain to the CML assay medium (cf. Fig. 3). However, Ukrain also induced a significant and dose-dependent increase in the lytic activity of control spleen cells maintained *in vitro* in the absence of IL-2. The Ukrain-induced increase in lytic activity of both spleen cell populations was not significantly different from each other. Thus, regardless of whether *in vivo* primed spleen cells were further treated *in vitro* with IL-2 or not, the addition of Ukrain to the CML assays, produced maximal activation of specific lytic activity, which was 60.0 ± 5% at 1.18 μM. Taking in consideration that peritoneal exudate lymphocytes with endogenous lytic activity of 70.0%, could not be further increased by the addition of Ukrain to the CML assays (data not shown), suggests that this alkaloid derivative induces the expression of the maximal lytic activity attainable by the effector cell populations used in these studies. To our knowledge, we are not aware of any other BRM capable of direct *in vitro* activation of a latent lytic potential of *in vivo* primed effector cells.

The results from the present studies demonstrate that the alkaloid derivative Ukrain is an effective BRM, in that it directly activates the lytic mechanism of immune effector cells. The enhanced lytic activity of spleen and peritoneal exudate lymphocytes retained specificity for the alloimmunizing tumor cells. Significantly, lymphocytes from alloimmunized mice, which had no significant endogenous lytic activity, displayed the greatest increase in target specific lytic activity, i.e. a 48-fold increase, by the addition of Ukrain to the CML assay medium. These results provide the first insights as to the possible mechanism by which Ukrain may achieve its therapeutic effectiveness in cancer patients (Nowicky *et al.*, 1991).

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