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THE *IN VITRO* POTENTIATION OF LAK CELL CYTOTOXICITY IN CANCER AND AIDS PATIENTS INDUCED BY F3 — A FRACTIONATED EXTRACT OF ASTRAGALUS MEMBRANACEUS

Chu Datong 储大同 Lin Juanru* 林绢如 Wong Wendy* Mavligit Giora*

*Cancer Hospital, Chinese Academy of Medical Sciences, Beijing 100021; *M.D. Anderson Cancer Center, The University of Texas, Houston, Texas 77030*

The *in vitro* induction of LAK cell activity was studied in cancer and AIDS patients. F3, an immunoregulatory component of Astragalus membranaceus was shown capable of potentiating LAK cell activity induced by rIL-2. The LAK cells killing activity against Hs294T melanoma cell line induced by 50 U/ml rIL-2 in the presence of F3 (55 μg/ml) reached 64%, which was comparable to that (60%) induced by 500 U/ml of rIL-2 alone. With F3 and rIL-2, the effector to target ratio could be reduced to one-half in order to obtain an equivalent level of cytotoxicity induced by rIL-2 alone. In some patients whose peripheral blood lymphocytes were relatively inert of rIL-2, F3 could make them responsive to rIL-2 induction. These results imply that F3 may be useful to potentiate LAK cell activity, reduce the dosage of rIL-2 and thus minimize the later's toxic side effects when used *in vivo*.

Key word: astragalus membranaceus, LAK cells, rIL-2.

It has long been a problem that the capillary leaking syndrome, fluid retention, myocardial infarction and arrhythmias caused by high dosage of rIL-2 infusion have not been effectively solved.[1-3] Therefore, the real value of rIL-2 plus LAK cell therapy in clinical practice clearly entered the stage of more objective evaluation.[4] Our previous work demonstrated that fraction 3 (F3) derived from a Chinese traditional medicinal herb —Astragalus membranaceus could greatly potentiate the LAK cell cytotoxicity induced by low dose of rIL-2 in normal donors.[5] Whether F3 could retain the same efficacy in cancer and AIDS patients is a worthwhile issue to be explored. Based on this consideration, further investigation has been done in our laboratory.

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MATERIALS AND METHODS

Subjects

This group of patients were all from M.D. Anderson Cancer Center and without any prior treatment for at least four months. 12 cases includes male 9, female 3. Their age ranged from 22–56 years with an average being 38.2. Among them, 4 cases were malignant melanoma with liver metastasis, 3 cases for colon cancer, 3 cases for AIDS with Koposi's sarcoma and 2 cases for AIDS related complex (ARC).

Tumor Cell Lines

Two human melanoma cell lines, A375P and Hs294T[6-8] (from the department of clinical immunology and biological therapy, M.D. Anderson Cancer Center) were cultured in 75 cm^2 tissue culture flasks

(Costar) containing minimum essential medium (MEM) with 10% fetal bovine serum, L-glutamine (2 mM) and gentamycin (50 μg/ml). The cells were incubated at 37 ℃ in a humidified 5% CO_2 incubator and were split every 3–5 days in order to maintain a proper cell density ($\leq 10^6$/ml). The passage of cell lines for the experiments usually were under 25. For each experiment, the cells were removed from the flasks by tripsinization, using 10 ml of Dulbecco's phosphate buffered saline solution (PBS) containing trypsin-EDTA (100 μg/ml). After five minutes of incubation, the cells were detached and washed once in complete MEM. They were then counted and placed into 96-well plates in a concentration of 5×10^3 cells per well. The plates were incubated at 37 ℃ in a humidified 5% CO_2 for three days prior to the addition of LAK cells.

LAK Cell Generation and Preparation

Cancer, AIDS and ARC patients' circulating mononuclear cells were isolated from heparinized peripheral blood by Ficoll-Hypaque density gradient centrifugation and washed thrice with RPMI-1640 for the purpose of LAK cell generation. The pellet was resuspended in RPMI-1640 containing 50 μg/ml of gentamycin, 2 mM of L-glutamine and 10% FBS. Cells were readjusted to a final concentration of 1×10^6/ml with the same medium and placed in 25 mm^2 flasks (Cornings) supplemented with fixed (100 U/ml) or varying (5–2000 U/ml) concentrations of rIL-2 (Cetus Corporation). Half of the rIL-2 supplemented LAK cell flasks were additionally supplemented with varying (7.3–110 μg/ml) or fixed (55 μg/ml) concentrations of F3. All flasks were then incubated at 37 ℃ in a 5% CO_2 incubator for three days for LAK cell generation. The putative LAK cell suspensions were then washed twice with MEM and resuspended to a appropriate concentration before application in a cytotoxic assay.

Cytotoxicity Assay

The cytotoxic activity of the putative LAK cells was assessed by adding effector (LAK) cells to the plates containing target tumor cells in varying efector: target cell ratios and coincubating the mixtures at 37 ℃ for 24 hours. In addition to the experimental groups, controls included (a) tumor cells cultured alone; (b) tumor cells cultured with F3; (c) tumor cells cultured with F3-treated mononuclear cells; (d) tumor cells cultured with untreated mononuclear cells; (e) putative LAK cells (treated with rIL-2 alone) cultured without tumor cells; and (f) putative LAK cells (treated with rIL-2 plus F3) cultured without tumor cells. At the end of the 24 hours coincubation, the supernatant of each well was removed and the plates were washed twice with PBS. To evaluate the cytotoxicity, we used a quantitative colorimetric methodology, the Bio-Rad protein assay.[9] Briefly, the amount of protein measured by this assay reflects directly, the number of residual surviving tumor cells present in the plates when compared to controls. The protein content of these residual, adherent, tumor cells was determined following cell lysis induced by adding 50 μl of 10% ethanol to each well, shaking the plates on a platform for 10 minutes, and then freezing them at -80 ℃ for 30 minutes. This was followed by thawing at 37 ℃ for 15 minutes. The freeze/thaw procedure was repeated and then 200 μl of 1:5 dilution dye-reagent was added to each well and the protein content of each well was measured by reading the optical density using a multiwell scanning spectrophotometer (Dynatech MR 600 Micro ELISA Reader). Statistical analysis was done by the student's *t*-test.

RESULTS

The Influence of F3 in LAK Cell Activity Induced by rIL-2

The Hs294T tumor-cell killing (% lysis) by putative LAK cells (E:T ratio 60:1) generated by different concentrations of rIL-2 is shown in Figure 1. In the absence of F3, a minimum concentration of 25 U/ml of rIL-2 was required during the generation of LAK cells for demonstration of any tumor-killing activity (10%). By escalating the concentration of rIL-2, a dramatic increase in tumor-cell killing activity was observed, i.e., a 32% lysis at 100 U/ml and 61% lysis at 500 U/ml of rIL-2. When F3 was present during LAK cell generation with rIL-2, a marked increase in tumor-cell killing activity was observed at all points of rIL-2 concentration. The most significant increase in percent tumor-cell lysis occurred within the range of 10, 25, 50, and 100 U/ml of rIL-2, with an augmentation factor of 10.2, 5.1, 3.3, and 2.5 × respectively. With rIL-2 concentrations >100 U/ml,

the F3- mediated augmentation in tumor-cell killing reached a plateau. Considering the sustained possible serum level of rIL-2 in the clinical practice, it is also evident (Figure 1) that the tumor-cell killing activity of LAK cells, generated with F3+50 U/ml of rIL-2 (64%) is equivalent to that (60%) resulting from LAK cell generation by 500 U/ml of rIL-2 alone. It is a ten folds reduction in the dosage of rIL-2 and the same phenomenon was observed with F3+LAK cells generated from AIDS and ARC patients.

The Effect of LAK Cell Generation in the Presence of F3 on the E:T Ratio Requirement for Tumor Cell Lysis

The result is shown in Table 1. Using two melanoma cell lines and a fixed concentration of 100 U/ml of rIL-2, LAK cell activity, expressed as % tumor cell lysis, is clearly related to the effector:target cell ratio. Thus, with rIL-2 alone, the mean percent cell lysis for T:E ratio of 1:60 was 25.7±13.6% and 32.8±13.6 respectively (for the two cell lines), compared to 17.1±12.2% and 21.7±12.4% for T:E ratio of 1:30, $P<0.01$ and $P<0.01$ respectively.

Table 1. Potentiation of LAK cell cytotoxicity in cancer and AIDS patients induced by F3 (% of tumor cell lysis)

Pt [a]	Diagnosis	Reagents added to MNC	A357P (%)		Hs294T (%)	
			T:E=1:60	T:E=1:30	T:E=1:60	T:E=1:30
1	Melanoma liver mets	rIL-2*	15	9	38	20
		rIL-2+F3**	25	12	67	45
2	Colon Ca	rIL-2	51	45	58	46
		rIL-2+F3	65	55	80	63
3	Colon Ca	rIL-2	45	33	50	38
		rIL-2+F3	77	56	79	59
4	Melanoma liver mets	rIL-2	38	30	46	35
		rIL-2+F3	51	43	65	57
5	Colon Ca	rIL-2	17	8	32	17
		rIL-2+F3	56	15	68	39
6	Melanoma liver mets	rIL-2	13	10	19	10
		rIL-2+F3	67	38	66	32
7	Melanoma liver mets	rIL-2	19	12	22	11
		rIL-2+F3	62	29	54	35
8	ARC	rIL-2	38	17	42	30
		rIL-2+F3	74	43	80	59
9	ARC	rIL-2	19	11	23	15
		rIL-2+F3	49	31	56	40
10	AIDS	rIL-2	13	7	19	10
		rIL-2+F3	45	23	51	30
11	AIDS	rIL-2	15	9	20	13
		rIL-2+F3	42	27	51	33
12	AIDS	rIL-2	25	14	24	15
		rIL-2+F3	57	34	59	41
	$\bar{x}\pm s$	rIL-2	25.7±13.6 *	17.1±12.2 *	32.8±13.6 *	21.7±12.4 *
			$>^{\Diamond}$	$>^{\Diamond}$	$>^{\Diamond}$	$>^{\Diamond}$
		rIL-2+F3	55.8±14.6	33.8±13.9	64.7±10.8	44.4±11.9

Note: *rIL-2 with a fixed concentration of 100 μ/ml for all cultures. **F3 with a fixed concentration of 55 μg/ml carbohydrate. $^{\Diamond}P<0.001$, $^{**}P<0.01$

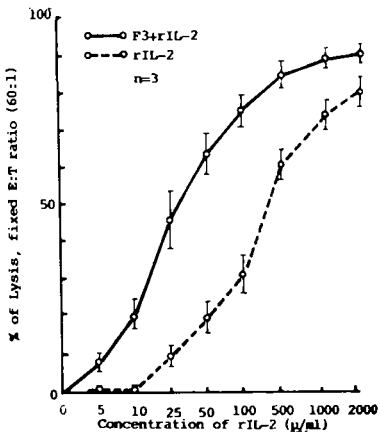

Fig. 1. Potentiating effect of F3 on rIL-2 induction of LAK cells' cytotoxic activity against Hs294T melanoma cell line

When the generation of LAK cells took place in the presence of both rIL-2 and F3, the percent tumor cell lysis was increased significantly, for both T:E of 1:60 and 1:30. It is also noteworthy, that the increased values of percent cell lysis for LAK cells generated in the presence of F3, in a less-favorable T:E ratio of 1:30 (i.e., 33.8±13.9% for A375P cell line and 44.4±11.9% for Hs294T cell line) completely reached or even exceeded the level generated with rIL-2 alone in a more favorable T:E ratio of 1:60. It convincingly shows that once F3 was added, even half of the effector cells could maintain the same killing intensity.

It is also of interest that in experiment #6, #10, and #11, rIL-2 alone was incapable of generating LAK cell activity, irrespective of the target cell line or T:E ratio used. This apparent, albeit sporadic, resistance to LAK cell induction by rIL-2 was completely overcome by co-incubation with F3, particularly for the T:E ratios of 1:60.

The Influence of Different Concentrations of F3 in the Induction of LAK Cell Activity

It is shown in Table 2. With a fixed concentration of rIL-2 (100 U/ml), the killing activity induced by LAK cells (without F3) was 29.5±8.8% in Hs294T cell line. Once different concentrations of F3 was added, the cytotoxicity increased in all experimental groups, i.e., 44.2±7.1% of tumor cell lysis by 7.3 μg/ml of F3 ($P<0.05$). The optimal concentration of F3 in the induction of LAK cells was 55 μg/ml with the killing activity being 71.8±10.3%. When compared with the control group induced by rIL-2 alone, the significance of statistical analysis was $P<0.001$.

Table 2. Potentiation fo LAK cell cytotoxicity induced by different concentrations of F3 (% of Hs294T melanoma cell lysis)

Exp. no.	LAK cell alone	LAK cells induced by F3 (μg/ml)				
		7.3	11	22	55	110
1	31	46	53	60	69	71
2	29	42	49	63	77	80
3	38	53	60	71	82	79
4	40	51	59	68	80	81
5	20	36	41	50	54	56
6	19	37	43	54	69	63
$\bar{x}\pm s$	29.5±8.8**	44.2±7.1*	50.8±7.8	61.0±8.0	71.8±10.3*	71.7±10.3

Note: LAK cells was induced by a fixed concentration of 100 U/ml of rIL-2. E:T=60:1
$^{*}P<0.001$, $^{*}P<0.05$

DISCUSSION

In late 1985, a sensational news from New England Journal of Medicine reported by Stephen Rosenberg and his colleagues[10] attracted a big attention among the international medical society, in

which a 44% of response rate was achieved in 25 cases of advanced renal cell carcinoma and malignant melanoma treated with high dosage of rIL-2 and LAK cells. Since then, a new approach of bio-immunotherapy was initiated in a growing scale. In 1987, Rosenberg summarized the results of 157 cases of malignant tumors with the response rate being 31%.[11] Again in 1989, the response rate of 652 cases decreased to 25%.[12] Meanwhile, he pointed out that the limitation of this therapy was severe capillary leaking syndrome caused by high dosage of rIL-2 infusion. From then, the repetitive work of several institutes and cancer centers around the world revealed that the response rate only ranged from 20–25%[13] and the side effects were difficult to be overcome. Therefore, the evaluation of this therapy entered more objective and cooling stage.[3,4,14]

The immunodeficient status of AIDS or ARC has bothered the medical care people for a long time. It is obvious that the amount of lymphocytes, the ability of being activated by various cytokines, and the potency of these cells to attack pathogens within the body are closely related to the efficacy of anti-infections in AIDS and ARC patients.

Our experiment showed that in both groups of tumor cell lines, after F3 induction, the cytotoxicity of LAK cells from AIDS and cancer patients increased significantly. It is manifested by the demonstration of an equivalent level of tumor cell lysis effected with (a) 10-fold lower concentration of rIL-2 during the stage of LAK cell generation or (b) with half the T:E ratio during the performance of the cytotoxic assay. In addition, due to F3 co-stimulation, some insensitive cases in rIL-2 induction become very sensitive. It is an attractive issue whether some patients inert to rIL-2 therapy could get benefit from the future clincal trial.

Our previous work showed that F3 could greatly increase the cytotoxicity in the sub-group of patients with low NK cell function, especially under the condition of co-existance of physiological level of IL-2.[15] Again, in a graft-versus-host reaction animal model, F3 could greatly enhance the killing activity of T cytotoxic cells derived from immune suppressed rats.[16] The results of these study suggest that among nonspecific LAK phenomenon, F3 may increase the killing activity of specific T cytotoxic cells, therefore the attacking potency of LAK cells was increased.

The issue of immunopharmacologic attenuation of toxicity, with retention of biological activity by the concomitant use of another natural biological response modifier, along with lower doses of rIL-2 is attractive and should be further pursued, especially during the period that the high dose of rIL-2 plus LAK cell therapy underwent crucial test.

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