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ADOPTIVE IMMUNOTHERAPY OF EXPERIMENTAL TUMORS USING CYTOTOXIC LYMPHOCYTES TO CARRY AND DELIVER TOXINS

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Summary. - *Adoptive transfer of specifically sensitized lymphoid cells constitutes a potential tool for specific cancer immunotherapy, however, the requirement of syngeneic or autologous specifically reactive cells has limited its use in the treatment of human cancer. In several mouse tumor models, large amounts of lymphokine activated killer (LAK) cells associated with high doses of interleukin 2 (IL-2) are able to mediate the regression of established pulmonary and hepatic metastases. Since LAK cells are more easily generated then specifically sensitized lymphocytes and show broad tumor specificity, this approach has been applied in humans to treat cancer, but the acute toxicity associated with the high doses of IL-2 administered represents an important drawback to its clinical use. The observation that lymphocytes can internalize ricin, a toxic plant protein, and then release it in an active form, capable of destroying other cells, led us to investigate the possibility of using antigen-specific cytotoxic T lymphocytes (CTL) or LAK cells as carriers of toxic substances to the tumor site. In our experimental models we observed that tumor-specific CTL or LAK cells can be used to deliver ricin into the tumor mass and cause temporary tumor growth inhibition.*

KEY WORDS: cancer immunotherapy, cytotoxic lymphocytes, ricin.

Riassunto (Uso di linfociti citotossici come veicoli di tossine nell'immunoterapia adottiva di tumori sperimentali). - *Il trasferimento adottivo di linfociti presensibilizzati verso un antigene costituisce un potenziale approccio per l'immunoterapia dei tumori, la cui applicazione nel trattamento delle neoplasie umane è limitata dalla necessità di trasferire un elevato numero di cellule singeniche o autologhe, antigene-specifiche. In alcuni modelli di tumori murini il trattamento con alte dosi di cellule LAK ed IL-2 si è dimostrato in grado di indurre regressione di metastasi a localizzazione polmonare o epatica; ma gli effetti tossici associati alla somministrazione di alte dosi di IL-2 limitano l'applicazione in campo clinico anche di questo secondo approccio terapeutico. L'osservazione che linfociti splenici murini, incubati con ricina, sono in grado di internalizzare questa tossina e successivamente di rilasciarla senza modificarne l'attività tossica, ci ha indotto ad esaminare la possibilità di utilizzare CTL antigene-specifici o cellule LAK come veicoli di ricina nella sede del tumore. Utilizzando due diversi modelli sperimentali abbiamo osservato che cellule citotossiche pretrattate con ricina sono capaci di indurre una temporanea riduzione della crescita tumorale sia di neoplasie a localizzazione periferica che di metastasi polmonari.*

PAROLE CHIAVE: immunoterapia del cancro, linfociti citotossici, ricina.

Introduction

Various immunotherapeutic approaches to the treatment of tumors have been considered over the past several decades. The exact role of the immune system as a surveillance and effector mechanism against neoplasms is still controversial, but recent progress in immunology and biotechnology have greatly contributed to the development of novel experimentation in the area of cancer therapy. In particular, advances in cellular immunology and molecular genetic techniques have led to new strategies in adoptive immunotherapy, i.e. local or systemic transfer of immunologically active cells into a tumor-bearing recipient host. The adoptive immunological intervention with immune lymphocytes has several theoretical advantages over the active immunization approaches; specificity and sensitivity are higher, immunosuppressive effects are absent, and ready combination with other therapies is possible.

Animal models have played an important role in the development of adoptive immunotherapy, and several types of lymphocytes were transferred in attempts to

inhibit tumor growth. Much initial work consisted of the transfer of lymphoid cells from animals, that were immune to tumor-specific transplantation antigens, into syngeneic recipients bearing an antigenically-related tumor [1]. These attempts demonstrated that established tumor transplants could be eliminated by the passive transfer of high numbers of sensitized cells. In addition, depending on the experimental system, $CD8^-$, $CD8^+$ or a mixture of both these cell populations were required [2]. Advances in tissue culture techniques, as well as the availibility of large amounts of recombinant lymphokines, such as interleukin 2 (IL-2), led to the primary sensitization of T cells *in vitro* and their expansion with the possibility of clone generation [3-5]. T cell lines and clones, which maintain their ability to kill tumor cells specifically, were expanded to large numbers *in vitro* and then used in adoptive therapy of disseminated tumors [6]. Since these cultured T cells were dependent upon IL-2 for survival, the efficacy of infusing exogenous IL-2 after cell transfer was explored. From these studies it emerged that the administration of exogenous IL-2 in combination with T cell transfer could promote the proliferation of adoptively transferred lymphoid cells, and thus heighten the therapeutic efficacy of cultured T cells [7]. Nevertheless, this therapeutic approach has received little attention in human cancer therapy, mostly due to the difficulty in obtaining lymphocytes with selective anti-tumor activity. Moreover, the requirement of large numbers of autologous lymphocytes for transfer into tumor-bearing patients constitutes a further obstacle to the development of successful adoptive immunotherapy in man.

In 1980, Rosenberg *et al.* first described the generation of cytotoxic cells following short-term stimulation of normal lymphocytes in IL-2-containing medium [8]. The resulting cells, defined lymphokine-activated killer (LAK) cells, were able to lyse a broad spectrum of targets, including fresh syngeneic and allogeneic NK-resistant tumor, but not normal cells [9]. LAK cells are readily generated in large amounts, both in experimental animals as well as humans [10], and display characteristics that better satisfy the requirements of cancer immunotherapy, comprehending the rapid acquisition of the capacity to kill tumor cells without prerequisite of sensitization with antigen, as well as the ability to kill a broad spectrum of targets including the poorly immunogenic tumors. Moreover, the adoptive transfer of large numbers of LAK cells in combination with the systemic administration of IL-2 was able to mediate the regression of established pulmonary and hepatic metastases in several mouse tumor models [11, 12]. The rationale of this therapeutic approach is based on the *in vivo* expansion of the injected cells under the influence of IL-2.

The efficacy of LAK cell adoptive therapy in humans is not yet entirely clear. Utilizing a regimen consisting of high-doses of IL-2 associated with LAK cell transfer, a therapeutic effect was demonstrated in patients with advanced renal cell carcinoma or melanoma [13, 14]. But in recent and more extensive clinical trials, controversial responses were obtained. In addition, the acute toxicity associated with the high doses of IL-2 administered represents an important drawback to its clinical use. Recently, tumor-infiltrating lymphocytes (TIL) were shown to acquire high cytotoxic activity after stimulation with IL-2, and a method for their large-scale *in vitro* propagation was developed [15]. However, tumor-specificity studies showed that these cells frequently did not display a pattern of specific autologous tumor cell killing [16]. Thus, the original goal of selective propagation of highly specific T lymphocytes obtained from autologous tumors was not achieved by the current TIL culture system. In this regard, the TIL from malignant melanoma might constitute an exception, since it was reported that they exhibit a high specificity for tumor-related antigens [17].

All the above preclinical and clinical immunotherapeutic interventions have a major disadvantage, consisting of an altered *in vivo* traffic of transferred cells [18, 19]. Several events hamper the transferred cells from homing specifically to the tumor site and surviving in the recipient donor. In particular, following i.v. injection, many lymphoid cells are immobilized in the lung capillaries, and in part destroyed. This phenomenon is particularly striking when cells maintained *in vitro* are used. Such cell "trapping" may be facilitated by several factors: low pulmonary circulation pressure; transient narrowing of the capillary bed during expiration, and cell alterations due to *in vitro* manipulations [20]. The altered *in vivo* traffic of transferred cells, on one hand, may limit the number of effector cells that selectively accumulate within peripheral tumors, but on the other, it facilitates their localization in organs such as the lung and liver which are frequently subject to metastases. In view of the serious limitations observed in each of the approaches described above, alternative strategies are now addressing towards the increasing activity of the transferred cells, so that lower numbers of highly efficient cells together with lower doses of IL-2 might be sufficient for an optimal anti-cancer effect. Such a strategy should eliminate the toxicities associated with IL-2 infusion and heighten therapeutic efficacy.

A promising procedure for enhancing the anti-tumor activity of transferred cells was recently developed in our laboratory.

Ricin uptake and excretion by CTL and LAK cells

The observation that lymphocytes can internalize ricin, a toxic plant protein, and then release it in an active form, capable of destroying other untreated cells [21], prompted us to explore the possibility of using antigen-specific cytotoxic CTL or LAK cells to carry toxin to the tumor site.

Preliminary *in vitro* experiments to evaluate ricin uptake and kinetics of release showed that following incubation for 1 h with 10 μg of ^{125}I-labelled toxin, tumor-specific CTL clone or LAK cells internalized large amounts of ricin (2.7 and 0.8 pg for 10^2 CTL clone and LAK cells, respectively), most of which was rapidly released into the medium during the first 30 min, and thereafter at a steady but

lower rate [22]. The ricin-treated cells were still highly viable, and could bind and lyse target cells several hours after loading, despite a gradual loss in cytotoxic activity.

The toxicity of the released ricin was studied by co-culturing loaded cells with different tumor cell lines, and then determining tumor cell proliferation after 48 h. When ricin-pretreated CTL clone cells were added to cultures, antigen-related MBL-2 cells, as well as antigen-unrelated P815 cells showed growth inhibition, which indicates that the released ricin is still toxic [22]. In similar experiments, ricin-loaded LAK cells were co-cultured with two tumor cell lines that are both lysed by LAK cells, but have a different susceptibility to ricin. This different pattern of sensitivity was confirmed by the finding that addition of ricin-treated LAK cells to cultures inhibited the growth only of the ricin-sensitive tumor cell line, and had little effect on the ricin-resistant one.

In vivo distribution of ricin-treated cells

To determine the behaviour pattern of ricin-treated cells following their injection into recipient animals, ^{51}Cr-labelled, ricin-treated CTL clone or LAK cells were injected i.v. into sublethally irradiated mice, which were then sacrificed 1 or 6 h later. Up to 35% of the total radioactivity injected was contained in the lungs of mice sacrificed 1 h after cell transfer. No differences in localization between ricin-treated and untreated cells were observed, which demonstrated that ricin treatment did not modify the pattern of cell recirculation. After 6 h, radioactivity declined in the lungs and concomitantly increased in the liver; this change in cell distribution was more pronunced when ricin-treated cells were transferred. Since most of the transferred cells were trapped in the lungs and liver of recipient animals, it was calculated that a very low number (2%) of the *in vitro* cultured cells were able to localize in peripheral tumors after i.v. inoculation [23]. Tumor cell presence apparently did not modify the initial lung and liver trapping. In fact, we observed no preferential LAK cell accumulation in the lungs of mice injected i.v. with a LAK-sensitive tumor cell line (Fig. 1).

Anti-tumor activity of transferred ricin-treated cells

We previously observed that the i.v. transfer of high doses (2×10^7) of virus-specific CTL clone cells into syngeneic immunodepressed mice prevents the development of the tumors due to injection of Moloney-murine sarcoma virus (M-MSV) at a distant site [24]. We chose this tumor model to study the effects of adoptive transfer of ricin-treated virus-specific CTL in view of its high sensitivity to CTL activity. The i.v. transfer of low (0.5×10^6) doses of ricin-treated CTL clone cells was sufficient for a temporary inhibition of tumor growth, while the same dose of untreated CTL clone cells, or a comparable amount of free ricin were ineffective [22]. Transfer of higher cell doses (2×10^6) caused rapid death in the recipient mice, presumably due to ricin toxicity.

These results demonstrated that tumor-specific CTLs loaded with ricin can be used to deliver the toxin into the tumor mass where it causes temporary inhibition of tumor growth.

Since lungs and liver are the main localization sites of the transferred lymphocytes, it seemed reasonable to expect a good therapeutic effect against tumors metastatizing in these organs. Mice bearing lung metastases induced by previous i.v. injection of M4 (a sarcoma cell line originally induced by benzopyrene), or F1 (a melanoma cell line) cells, were injected with ricin-loaded LAK cells; the animals were killed 6-10 days later, and lung metastases were counted. Control groups consisted of mice injected with untreated LAK cells, free ricin, or sonicated ricin-treated LAK cells. We found that the injection of 7×10^6 ricin-treated LAK cells brought about a 70-80% decrease in the number of M4-induced metastases, while a non-significant reduction was observed in the control groups (Table 1). Analogous studies with the relatively ricin-resistant F1 cell line showed that i.v. injection of 2×10^5 F1 cells caused approximately 60 metastatic nodules in the lung 15 days later. Our previous *in vitro* observation of F1 cell resistance to ricin activity was thus confirmed by the *in vivo* finding of no significant decrease in the number of pulmonary metastases (Table 1).

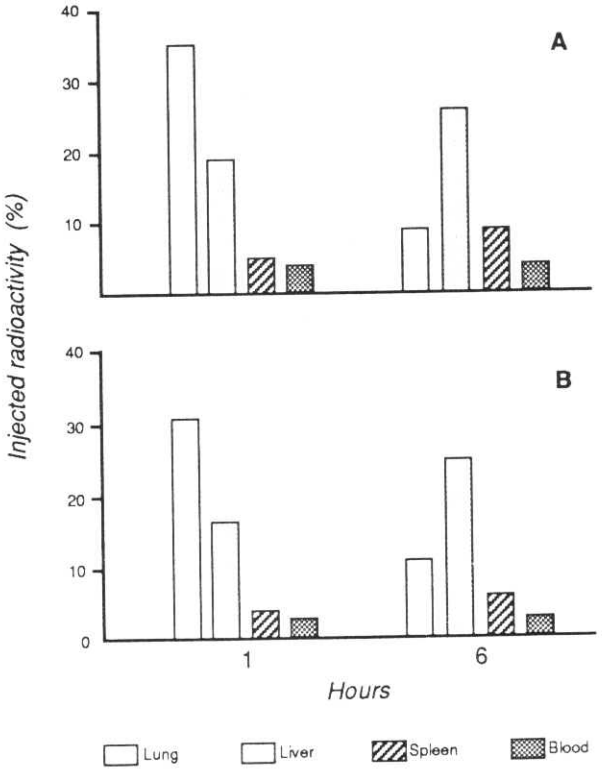

Fig. 1. - *In vivo* distribution of LAK cells 1 or 6 h after i.v. inoculation in syngeneic normal or tumor-carrying B6 mice. 2×10^6 ^{51}Cr-labeled LAK cells were injected into normal recipients (A) or in mice inoculated i.v., 4 days before, with 2.5×10^4 M4 cells (B). Results are expressed as percentage of injected radioactivity.

Table 1. - *Number of metastases in mice receiving an i.v. transfer of untreated or ricin-treated LAK cells*

Tumor cell line	Transferred cells (*)			
	None	Ricin alone	LAK cells	Ricin-treated LAK cells (**)
F1	58 ± 14	52 ± 9	41 ± 13	40 ± 21
M4	149 ± 25	130 ± 16	115 ± 32	45 ± 4

(*) 4 days after F1 (2×10^5) or M4 (2.5×10^4) tumor cell injection, recipient mice were injected i.v. with ricin (20 μg), with 7×10^6 untreated LAK or with 7×10^6 ricin-treated LAK cells. Ten days after tumor cell inoculation mice were killed and lung metastases counted.
(**) LAK cells were preincubated with 3 μg of ricin for 1 h.

Conclusion

The development of *in vitro* techniques enabling the activation and propagation of large numbers of immune lymphoid cells made adoptive transfer of autologous cells possible, and thus had important repercussions in the field of immunotherapy of human tumors. The generation of LAK cells, in particular, has provided a cell population with anti-tumor activity for clinical trials which are now exploring the efficacy of the systemic administration of both activated cells and IL-2.

Although *in vitro* cell expansion procedures have resolved the main drawbacks to adoptive immunotherapy approaches, they have also created new problems regarding transferred cell ability to traffic to the tumor site, and survive in the recipient organism. In fact, cell trapping in the lungs and liver limits the possibility of curing tumors arising in peripheral body sites, and therefore very recent studies are addressing the local/regional administration of combined LAK cells and IL-2 in the therapy of primary tumors. In reference to the problem of poor *in vitro* activated cell survival, the IL-2 doses needed to promote *in vivo* LAK cell expansion are extremely high, and are responsible for most of the toxic effects inherent in this therapeutic procedure.

In our experimental models we observed that tumor-specific CTL or LAK cells can be used to deliver ricin into the tumor mass and cause temporary tumor growth inhibition. Moreover, despite a relatively small decline in cytotoxic cell potential following incubation with ricin, *in vivo* anti-tumor activity was enhanced by the ricin pretreatment since low numbers of toxin-carrying cells were sufficient to inhibit tumor growth for several days. In addition, it is noteworthy that the amount of ricin delivered by the transferred cytotoxic cells was very close to the free ricin dose that proved to be lethal, as all the mice died 2 or 3 days after its i.v. introduction. So it seems reasonable to assume that cell delivery of ricin attenuates its systemic toxicity. Clearly, ricin's high toxicity continues to constitute a major drawback of this approach to tumor therapy, and further work is needed to optimize the protective effects seen. Nonetheless, the use of lymphoid cells as vehicles for drug delivery seems to have great possibilities. In particular, the entrapment of antineoplastic agents in tumor-specific cells may prove advantageous for achieving tumor-targeted drug release, and consequently, we may envisage a therapeutically effective drug concentration at the site of tumor growth with reduced toxic side effects. Similar studies in this direction were conducted using liposomes as drug or micromolecule carriers [25]. However, several attempts to increase the extent of lyposome interaction with target cells by inducing specific ligand receptor bindings have met with little success.

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