The use of gene transduction has considerably expanded the range of tools available for the immunotherapy of tumours. Protection from tumour growth in immunocompetent animals has been obtained with tumour cells transduced with genes for various cytokines and surface antigens (Tanaka et al., 1986; Ostrand-Rosenberg et al., 1991; Colombo and Forni, 1994). However, realistic therapeutic protocols, in which engineered cells are administered only after the challenge with live parent tumour cells, have been explored less frequently.

Malignant tumour cells transduced with foreign genes are a complex biological reagent. In fact, they should be regarded as a novel type of tumour, rather than as a passive immunological vaccine. In particular, some of the cytokines used for this type of studies are active both on the host immune system and on the tumour itself. In particular, interferon (IFN) gene transduction profoundly alters several properties of the transformed cells, including upmodulation of MHC, resistance to natural killer (NK) cells and metastatic ability (Watanabe et al., 1989; Gansbacher et al., 1990; Restifo et al., 1992; Lollini et al., 1993).

In principle, genes coding for class I major histocompatibility complex (MHC) should modify the interaction of tumour cells with the immune system only, but it has been clearly shown that after transfection of MHC genes a few properties of the neoplastic cells are also modified (De Giovanni et al., 1994).

These considerations suggest that the biological properties of recipient tumour cells must be adequately characterised, before and after gene transfer, to exploit the possibilities offered by this technology fully. In this work we show how to take advantage for therapeutic purposes of some specific modifications induced by transfection of the IFN-γ gene in a mammary carcinoma.

Materials and methods

Cells and DNA transfection

TSA-pc is a tumour cell line we derived from a spontaneous mammary adenocarcinoma of the BALB/c strain; TSA-pc cells give rise to moderately differentiated, non-capsulated invasive tumours, which are highly metastatic and poorly immunogenic in syngeneic mice (Lollini et al., 1993). TSA-pc has been used as the recipient of several cytokine genes (Colombo and Forni, 1994), including IFN-γ (Lollini et al., 1993). TSA-IFNγ and TSA-IFNγ were obtained from TSA following gene transfection with the murine IFN-γ gene and release 500 and 6000 IU ml⁻¹ of IFN-γ (Lollini et al., 1993); TSA-neo control was transfected with the neomycin resistance gene alone (Lollini et al., 1993). Clone TSA-IFNγ was subsequently transfected with allogeneic H-2Kb and H-2Dd genes (Lollini et al., 1995) to obtain clones designated TSA-IFNγ-Kb or TSA-IFNγ-Db. Hygromycin resistance was used to select transfectants; clone TSA-IFNγ-hygro was transfected with the hygromycin resistance gene alone. Cells were cultured in Dulbecco's modified eagle medium (DMEM; Gibco, Paisley, UK), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco); cultures were maintained at 37°C in a humidified atmosphere of 5% carbon dioxide in air. All the cells employed were >90% viable as judged by trypan blue dye exclusion.

Mice and TSA-pc challenge

Seven-week-old female BALB/cAnNCrlBR (BALB/c) mice and 4-week-old female mu/mu mice on Swiss CD-1 back-
ground were purchased from Charles River Laboratories (Calco, Italy) and treated according to European Community guidelines. Mice were allowed to rest for 1 week before any treatment. Metastases were induced by i.v. injection of TSA-pc cells in a lateral tail vein (5 × 10⁶ cells in BALB/c mice or 5 × 10⁵ cells in nude mice). Lung nodules were evaluated at day +21; all metastasis counts were performed on dissected lung lobes contrasted with black India ink under a stereoscopic microscope.

**Treatment**

Either before or after the TSA-pc challenge, mice received transfectants treated with 60 μg ml⁻¹ mitomycin-C (Sigma, Milan, Italy) at 37°C for 45 min to abolish their residual tumorigenicity (Allione et al., 1994). Prophylactic vaccinations consisted of a single injection of 1 × 10⁶ mitomycin-C-treated (MitC) cells performed 30 days before challenge. Therapeutic vaccinations started 1 day after TSA-pc challenge and consisted of six injections of 1 × 10⁶ MitC cells 3–4 days apart.

**In vitro cytotoxicity tests**

Cytotoxicity was tested using lymph node (axillary, inguinal and mesenteric) cells or macrophages from mice repeatedly immunised with 1 × 10⁶ MitC cells s.c. In some experiments, effectors were restimulated by a 6 day *in vitro* coculture with MitC TSA-pc cells and then tested for cytotoxic activity. Target cells were labelled by incubating non-confluent monolayers in 25 cm² tissue culture flasks for 24 h with 5 ml of medium containing 1 μCi ml⁻¹ [³H]thymidine (26 Ci mmol⁻¹; NEN, Milan, Italy). Target cells were then harvested by trypsin–EDTA (Gibco) treatment, washed twice and resuspended to a concentration of 5 × 10⁴ cells ml⁻¹ in the test medium. Effector cells were admixed in triplicate with 5 × 10⁴ labelled target cells at 50 : 1, 25 : 1, 12 : 1 and 6 : 1 E:T ratios in round-bottomed 96-well microtitre plates. After 48 h incubation at 37°C in 5% carbon dioxide, 0.1 ml of the supernatant was taken and the percentage of specific lysis was calculated as previously described (Lollini et al., 1993). In some experiments, ¹²⁵I-labelled TSA-pc cells were also used to evaluate 4 h release in the presence of effectors obtained as above, but very low values of cytotoxicity were obtained owing to the resistance to lysis of TSA-pc target cells (data not shown). Maximum release was determined by adding 0.1 ml of 10% Triton X-100. Values of spontaneous release were within 10–15%. The release values are expressed as lytic units (LU) calculated as described (Lollini et al., 1993). One LU is here defined as the number of effector cells needed to kill 20% of the target cells (LU₂₀).

**Statistical analysis**

The non-parametric Wilcoxon's rank-sum test was used to compare numbers of metastases.

**Results**

**Immunogenicity of TSA-IFNγ clones**

TSA-pc is a highly malignant tumour, and expression of the IFN-γ gene did not abolish completely the tumorigenicity of clones TSA-IFN⁺⁺⁺ and TSA-IFN⁺⁺⁺⁺ (which release 500 and 6000 IU ml⁻¹ IFN-γ respectively), thus mitomycin-C-treated (MitC) cells were used for therapeutic administrations. MitC cells retain for several days the ability to release IFN-γ, as shown by reverse transcriptase–polymerase chain reaction and IFN-γ bioassay (data not shown).

We have previously shown that the reduced tumorigenic potential of IFN-γ clones was mainly caused by the local activity of macrophages. However, tumour infiltrate also contained activated lymphocytes (Lollini et al., 1993). IFN-γ clones induced memory cells directed against the parent tumour TSA-pc; mice immunised with IFN-γ clones were protected against a subsequent challenge with TSA-pc (Musiani et al., 1994). Mice immunised with TSA-IFN⁺⁺⁺ or TSA-IFN⁺⁺⁺⁺ displayed a stronger cytotoxicity against TSA-pc than mice immunised with TSA-pc or TSA-neo (Figure 1).

**Figure 1** Lysis of TSA-pc by restimulated lymph node cells from BALB/c mice immunised s.c. in the right thigh with 10⁶ MitC cells.

**Figure 2** Therapy of TSA-pc lung metastases with IFN-γ transfectants. BALB/c mice challenged with 5 × 10⁶ live TSA-pc cells i.v. received six injections of 10⁶ MitC cells s.c. in a thigh. (a) Metastasis-free mice/number of treated mice. (b) Range of lung metastases. *Number of metastases significantly different (P<0.01 at least) from untreated or TSA-pc-treated mice by Wilcoxon's rank-sum test.
Therapy of lung metastases

The ability of IFN-γ clones to enhance macrophage activity (Lollini et al., 1993) and to elicit memory cells suggests that these clones could be used for immunotherapy. Since a major goal of cancer immunotherapy is to prevent the development of overt metastases in tumour-free hosts, BALB/c mice were first challenged i.v. with TSA-pc cells and then repeatedly treated s.c. with MitC-TSA-IFNγ clones (Figure 2). Therapy with IFN-γ clones yielded more than one-third of mice free from lung metastases and reduced by 90% the median number of metastatic foci per mouse.

The lack of therapeutic efficacy of IFN-γ transfecant cells in nude mice (Figure 3), compared with immunocompetent hosts (see Figure 2), showed that an intact T-cell response is required for the cure of lung metastases.

Self-targeting of transfecants

The treatment of tumour cells with IFN-γ enhances their survival in the post-intravasation phases of the metastatic process, probably through an increase in resistance to NK cells (Kelly et al., 1991). TSA-IFNγ500 and TSA-IFNγ1000, even though they have a reduced tumorigenic potential compared with TSA-pc, are more metastatic after i.v. injection (Lollini et al., 1993). However, this higher metastatic capacity of TSA-IFNγ clones could be exploited for a selective delivery of IFN-γ-releasing cells to the lung, where TSA-pc metastasises.

Therefore, the therapeutic effect of IFN-γ clones administered i.v., a route which is not commonly used for immunotherapy in mice, was next evaluated. First, the immune memory against TSA-pc experimental metastasis elicited by MitC IFN-γ clones injected i.v. was evaluated. The preimmunisation (day −30) with either MitC-TSA-pc or IFN-γ clones did not elicit protection against a TSA-pc i.v. challenge (day 0) (data not shown). By contrast, when MitC-TSA-IFN-γ clones were administered i.v. after an i.v. TSA-pc challenge, a significant inhibition in the number of lung nodules was found (Figure 4). On the other hand, the same treatment with MitC-TSA-pc actually enhanced the development of metastases. Taken together, these results suggest that the IFN-γ released by transfecants in the lungs acts via inflammatory, rather than memory, components.

Therapy of lung colonies with IFN-γ plus H-2Kb double transfecants

The results with IFN-γ clones were encouraging, especially considering that TSA is a highly malignant tumour. However, to potentiate further the interactions of tumour cells with the immune system of the host, we retransfected TSA-IFNγ1000 cells with allogeneic H-2Kb or H-2Dd genes to exploit the high expression of IFN-induced molecules in TSA-IFN-γ clones. Moreover, since IFN-γ transfecants appeared to stimulate macrophages more than T cells, the addition of allogeneic MHC antigens could tip the balance in favour of a T response, mainly through the release of...
autocrine/paracrine helper cytokines by alloreactive lymphocytes.

The expression of allogeneic H-2\(^d\) antigens obtained after transfection of TSA-IFN\(_{\gamma}\)\(^{6000}\) was indeed very high (Figure 5). Similar transfections yielded only clones expressing low levels of H-2 antigens, when cells which do not produce IFN-\(\gamma\) were used as recipients (De Giovanni et al., 1994). Other in vitro growth characteristics of double transfectants did not differ from those of parent TSA-IFN\(_{\gamma}\)\(^{6000}\) cells. In vivo, both the tumorigenicity and the metastatic ability of double transfectants in immunocompetent mice were almost completely abolished (Lollini et al., 1995); both T cells and macrophages were found to be involved in the rejection of these cells. In nude mice the tumorigenic potential of double transfectants was similar to that of control or parental cells, thus confirming the role played by T cells (Lollini et al., 1995).

Lymphocytes of mice immunised with cells transfected with IFN-\(\gamma\) plus H-2 genes killed TSA-pc target cells even without in vitro restimulation (Figure 6), and their cytotoxic activity was 3–4 times higher than that of lymphocytes of mice immunised with TSA-IFN\(_{\gamma}\)\(^{6000}\). Unprimed macrophages showed a very high cytotoxic activity against all cells releasing IFN-\(\gamma\), regardless of allogeneic MHC expression (Lollini et al., 1995).

MitC double transfectant cells were used to treat mice that had received live TSA-pc cells i.v. (Figure 7). TSA-IFN\(_{\gamma}\)\(^{6000}\)-Db was more effective than the TSA-IFN\(_{\gamma}\)\(^{6000}\) clone. Moreover, the rejection of double transfectant tumours by the host enabled us to compare proliferating cells and MitC cells. When administered as proliferating cells, H-2\(^d\) transfectant cells were significantly more effective than TSA-IFN\(_{\gamma}\)\(^{6000}\). Note that the control cells shown in Figure 7, i.e. TSA-IFN\(_{\gamma}\)\(^{6000}\) and the hygro transfectant, could not be used without mitomycin treatment, since they gave rise to progressive tumours at the site of injection. No therapeutic effect by TSA-IFN\(_{\gamma}\)\(^{6000}\)-Db was observed in nude mice, confirming the requirement of an intact T-cell response for the cure of lung metastases (data not shown). No mouse treated with TSA-pc cells transfected with an allogeneic MHC gene alone was cured (data not shown). It should be noted that allogeneic MHC expression of double transfectants was 70 times higher than expression of cells transfected with the MHC gene alone in the absence of autocrine induction by IFN-\(\gamma\).

Discussion

TSA is a highly malignant and poorly immunogenic mammary adenocarcinoma spontaneously arisen in a BALB/c mouse (Lollini et al., 1993). TSA is quite refractory to conventional chemotherapy and immunotherapy, and reproduces many features of human mammary carcinomas (De Giovanni et al., 1988; Deabate et al., 1992), thus it is a realistic model of human neoplasms. TSA cells have been used as recipients for many genes relevant to gene therapy of tumours, and it is one of the few systems available for comparative studies (Colombo and Forni, 1994; Allione et al., 1994). In this paper we have shown that repeated injections of MitC IFN-\(\gamma\) and allogeneic MHC class I gene-transduced TSA cells were effective in inhibiting lung metastases.

IFN-\(\gamma\) receptors are ubiquitous, thus gene therapy with IFN genes will bring about phenotypic modifications of the target cell along with immunomodulation of the host. We have shown here that the autocrine effects can be rationally exploited to devise specific therapeutic modalities. Tumour cells transfected with the IFN-\(\gamma\) gene, as well as cells treated with exogenous IFN-\(\gamma\), generally show a tremendous increase,
both in the expression of some IFN-sensitive genes, including MHC (Watanabe et al., 1989; Gansbacher et al., 1990; Chen and Ananthaswamy, 1993; Mizuno et al., 1994), and in the ability to colonise the lung after i.v. injection (Lollini et al., 1993). The latter is linked to the former via a decrease in sensitivity to NK cells (Kelly et al., 1991). Since TSA-IFN-γ transfectants maintain the selective homing to the lung of TSA-pc metastases, we injected MitC transfectants i.v. to obtain a selective delivery of IFN-γ to TSA-pc metastatic sites. In this way a significant therapeutic effect, but not immune memory, was obtained. Therefore, the function of TSA-IFN-γ clones in this setup was to act mainly as 'micropumps', effectively targeting the released IFN-γ to the lung and eliciting mainly a local response.

The results obtained after i.v. therapy indicated that a local stimulation of inflammatory cells could significantly reduce metastatic load, but no single mouse was completely free from metastases. Single and double transfectants administered s.c. showed a higher therapeutic efficacy, with 30–50% of mice free from detectable metastatic nodules. This supports the idea that engineered tumour cells elicit a cross-talk between non-specific effectors and T lymphocytes, leading to a rapid destruction of tumour cells at the site of cytokine release and to the establishment of a systemic, long-term T-cell response that can reach and destroy distant metastatic deposits (Colombo et al., 1992). Local tumour control can be effected by different inflammatory responses elicited by engineered tumour cells (Musiani et al., 1996); however, the expansion of an optimal T-cell reactivity is not always obtained (Colombo et al., 1992). Our results show that both the route of vaccination and the use of additional antigenic stimuli can tip the balance in favour of a curative T-cell response.

The autocrine stimulation of genes containing IFN response elements in IFN-γ transduced cells suggested that these cells could also be used as efficient recipients of IFN-sensitive genes. The allogeneic MHC genes, H-2Kb and H-2Dd, were used to confer upon IFN-γ transfectants an additional immunogenic signal. In the TSA model, IFN-γ induced mainly a local macrophage response (Lollini et al., 1993; Musiani et al., 1994); allogeneic MHC gene products stimulate many T lymphocytes, and double IFN-γ plus MHC gene transfectants were found to stimulate both macrophages and T lymphocytes simultaneously (Lollini et al., 1995). Moreover, IFN-γ plus MHC double transfectants were non-tumorigenic in immunocompetent mice, unlike IFN-γ single transfectants, and could be used as a live vaccine, which was therapeutically more effective than MitC cells, thus confirming our previous findings with TSA cells transduced with various cytokine genes (Allione et al., 1994).

Two issues deserve further discussion. Pleiotropic cytokines, such as IFN-γ, preferentially stimulate distinct immune response mechanisms in different tumour model systems. In effect, cells transduced with the IFN-γ gene were found to stimulate either T cells (Watanabe et al., 1989; Esumi et al., 1991; Teramura et al., 1993) or macrophages selectively (Gansbacher et al., 1990; Lollini et al., 1993; Hock et al., 1993), apparently in an unpredictable way. The IFN-γ and alio-MHC double transfection approach showed that, when IFN-γ-activated response depends on macrophages, the presence of allogeneic MHC elicits a T-cell response. The two MHC genes used in TSA transfectants did not yield identical results: H-2Dd was more effective than H-2Kb in the therapeutic setup, possibly due to the fact that H-2Kb transfectants are more rapidly rejected by the host (Lollini et al., 1995), and thus do not persist in vivo long enough to induce systemic immunity. The differential effect of H-2Kb and H-2Dd suggests that gene therapy approaches based on the transduction of allogeneic MHC genes may be further refined and optimised by selecting the most effective MHC regions and alleles, possibly on the basis of the MHC haplotypes of each patient.

Present data acquire particular importance, since several phase 1 clinical trials using gene-transduced tumour cells are in progress, while the real efficacy of this approach is still controversial (Colombo and Formi, 1994). About 50 different cell lines have been transduced worldwide with genes coding for IFN-γ; however, the number of studies actually dealing with therapeutic protocols is still quite small. The therapeutic efficacy obtained in our model was similar to that attained in the Lewis lung carcinoma system (Porgador et al., 1993); other studies with MBT-2 bladder carcinoma (Connor et al., 1993) and Dunning rat prostate carcinoma (Vieweg et al., 1994) reported a limited therapeutic success with IFN-γ-secreting cells. The studies present a number of common features: all used realistic models of malignant carcinomas; the therapeutic schedule comprised multiple administrations of similar doses of IFN-γ-transduced cells; and therapeutic vaccinations started when neoplastic deposits (either local or metastatic) were quite small. As a whole, these results suggest that a gene therapy approach based on IFN-γ is feasible and can be successful when the tumour load is small or metastatic deposits are still in the infancy of their natural history. Moreover, a rational exploitation of the biological properties of genetically engineered cells can further ameliorate the success rate obtained with the straightforward protocols adopted by earlier studies.

Finally, a crucial point of gene therapy with cytokine genes is which cytokine gives the best therapeutic results. No
common pattern has emerged from comparative studies conducted using different experimental model systems (Dranoff et al., 1993; Hock et al., 1993; Allione et al., 1994; Franco et al., 1994), but interferons, together with some interleukins and colony-stimulating factors, are clearly among the most promising candidates for this type of gene therapy. The early experimental work on cells transduced with genes coding for each individual cytokine aided in designing the currently ongoing phase 1 protocols. More extensive experimental studies are now required in which different genes, coding for cytokines or antigens, are compared in different tumor types, to obtain useful information for the design of phase II gene therapy studies in humans.

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