A Recombinant Single-Chain Antibody Interleukin-2 Fusion Protein

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ABSTRACT

Recombinant interleukin-2 (rIL-2) therapy has been shown to be of value in the treatment of some cases of melanoma and renal cell carcinoma. However, its use can be limited by severe systemic toxicity. Targeting rIL-2 to the tumor should improve the antitumor immune response and decrease the systemic toxicity. With this aim, we have employed recombinant DNA techniques to construct a single-chain antibody interleukin-2 fusion protein (SCA-IL-2).

The protein used in this model system consists of the variable domains of the antilysozyme antibody D1.3 fused to human IL-2 and is expressed in E. coli. It retains antigen-binding specificity and has the full biological activity of rIL-2.

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This approach can be taken to generate SCA-IL-2 proteins that bind to appropriate cellular antigens. In vivo administration of tumor-binding SCA-IL-2 should result in a localized high concentration of rIL-2 in the tumor tissues, maximizing the antitumor response while keeping systemic side effects to a minimum.

**Index Entries:** Bacterial expression; single chain antibody; fusion protein; interleukin-2.

**Abbreviations:** rIL-2, recombinant interleukin-2; SCA, single-chain antibody; SCA-IL-2, single-chain antibody interleukin-2 fusion protein.

**INTRODUCTION**

Interleukin-2 is a 15-kDa cytokine produced by T-helper cells that stimulates cytotoxic T-lymphocytes and NK cells (1). Bacterially produced recombinant IL-2 has been used clinically in the treatment of melanoma and renal cell carcinoma to stimulate cancer patients’ immune systems (2). Recent preclinical studies indicate that achieving a prolonged high dose of IL-2 in the tumor can result in the induction of a long-lasting antitumor response leading to the rejection of an otherwise lethal tumor (3). However, the in vivo efficacy is limited by difficulties in maintaining prolonged high doses in the tumor and by the severe systemic toxicity associated with high-dose IL-2 therapy (2).

It has been demonstrated that it is possible to incorporate IL-2 into a number of toxin-based fusion proteins while maintaining its partial or full activity (4-7), and a Fab'-IL-2 fusion protein produced in mammalian cells has been described recently (8). As a delivery system, the smaller single-chain antibody (SCA), comprising linked variable heavy (V_H) and variable light (V_L) chain antibody domains, shows great promise (9). Where tested, SCAs demonstrate good tissue penetration, rapid renal clearance of nonlocalized protein, and low immunogenicity (10). Recent advances with in vitro selection should allow the rapid and economic production of SCA of required specificity (11,12). In this study, we have fused amino acids 2-133 of human IL-2 to the carboxyl end of the antilysozyme SCA D1.3 and expressed the protein in E. coli. We demonstrate for the first time that this fusion protein, in addition to retaining antigen-binding specificity, possesses the full activity of IL-2.
when tested with lymphocytes bearing the high-affinity IL-2 receptor. Furthermore, the SCA-IL-2 fusion molecule described here retains full IL-2 action as measured by proliferative effects on human peripheral blood lymphocytes.

MATERIALS AND METHODS

Plasmid Assembly

Single colonies of E. coli containing plasmid pSV-HIL2-0 (gift of W. Friers, University of Ghent) were suspended in 500 μL of water, boiled for 5 min and cleared by centrifugation in a microfuge. Aliquots (10 μL) of the supernatant were subjected to polymerase chain reaction (PCR) amplification according to the manufacturer’s instructions (Perkin Elmer Cetus, Norwalk, CT) in the presence of 25 pmol each of oligonucleotide primers IL-2/7 and IL-2 3'. The reaction underwent 30 cycles of denaturation (94°C, 1 min), annealing (50°C, 1 min), and extension (72°C, 1 min). Oligonucleotide primer IL-2/7 (ACCAAGCTCGAGATCAAACGGGAACAAAAACTCCCTACTI'CAAGTTCT) directs the incorporation of an XhoI site and the seven carboxy-terminal amino acids of the D1.3 SCA plasmid (pSW2scD1.3myc [13], gift of E. S. Ward, LMB, Cambridge, UK) fused to sequences encoding amino acids 2-7 of human IL-2. Primer IL-2 3' (TTCTCGAATTCGAGCTGGATCCTTATTAAGTGTTGAGATGAT) directs the incorporation of an EcoRI site downstream of the termination codon of human IL-2. The 420-bp amplified product was isolated from a 1.5% agarose gel, digested with XhoI and EcoRI, and ligated between the XhoI and EcoRI, sites of plasmid pSWsFVD1.3myc to generate plasmid pSCA-IL-2/7. This plasmid bears a chimeric gene encoding a single-chain antibody-IL-2 fusion protein (SCA-IL-2) under lac transcriptional control. Figure 1 shows plasmids used, the nucleotide sequence, and the deduced amino acid sequence at the fusion junction.

Expression and Partial Purification of SCA-IL-2 Protein

Cultures (500 mL) of E. coli K12 KS476 (14) (gift of J. Beckwith, Harvard) transformed with plasmid pSCA-IL-2/7 were grown overnight at 37°C in 2 x TY broth supplemented with appropriate antibiot-
Fig. 1. Construction of the pSCA-IL-2/7 plasmid encoding the chimeric SCA-IL-2 fusion protein. Both plasmids are pUC 19 derivatives. Only the section between the HindIII and EcoRI sites is demonstrated.
After expression, cells were harvested, the bacterial growth supernatant was filtered (0.22-μm filter), and applied at room temperature to a lysozyme-Sepharose column. After washing with PBS, bound protein was eluted as described previously (15). Prior to use, the fusion protein was dialyzed exhaustively against PBS and stored at -20°C.

**ELISAs**

For serological detection of SCA-IL-2 fusions proteins, enzyme-linked immunosorbent assays (ELISAs) were employed. Flat-bottomed Dynatech Immulon 96-well plates were coated overnight at 25°C with hen egg lysozyme (300 μg · mL⁻¹), goat polyclonal anti-IL-2 antibody (50 μg · mL⁻¹, British Biotechnology Oxford), or other proteins (BSA, KLH, reconstituted milk powder, or insulin at appropriate concentrations) applied in 50 mM bicarbonate buffer, pH 9.6. Unoccupied sites were blocked with a 1% solution of milk powder in PBS for 30 min at 25°C. Bacterial supernatants, affinity-purified material, or recombinant IL-2 (rIL-2, Boehringer, Mannheim, Germany) were diluted in PBS/1% milk powder and incubated (30 min, 25°C) in appropriate wells. After three washes in PBS, bound protein was detected with either polyclonal anti-IL-2 or DMS1 (16), a murine monoclonal antibody that recognizes the receptor-binding site of human IL-2 (gift of K. A. Smith, Dartmouth Medical School, Hanover, NC). After a further three washes, bound antibody was detected with a species-specific HRP conjugated antibody (DAKO, Copenhagen, Denmark). ABTS was added to generate a color change that was monitored at 405 nm.

**Western Blots**

Affinity-purified protein samples were electrophoresed through 15% 30:1 acrylamide:bis-acrylamide gels essentially as described in Laemmli (17) and transferred electrophoretically to a nitrocellulose membrane (18). The membrane was blocked (30 min, 25°C) in a 1% solution of milk powder in Tris-buffered saline/0.0025% Tween 20 (TBST). Proteins were detected by incubation (1 h, 25°C) with either rabbit anti-SCA serum (gift of E. S. Ward) or mouse monoclonal DMS1. After five washes in TBST, bound first-step antibody was detected with antirabbit or antimouse AP conjugated antibody (Amersham, Bucks, UK) and revealed by incubation with a solution of NBT and BCIP (Promega, Madison, WI), according to the manufacturer’s recommendations.
Bioactivity Assays

CTLL-2 cells (19), which bear the high-affinity IL-2 receptor, were maintained in RPMI media supplemented with 10% fetal calf serum (heat inactivated) and 10 U·mL\(^{-1}\) rIL-2. For assay, cells were washed in media and deprived of IL-2 for 4 h, after which they were seeded into 96-well plates at \(5 \times 10^3\)/well. Dilutions of fusion protein or rIL-2 were added and the cultures incubated for 18 h at 37°C in a 5% CO\(_2\) atmosphere. Then to each well, 0.5 μCi of \(^3\)H-Thymidine (Amersham) was added. After a further 4 h of incubation, cells were harvested onto glass fiber filters, dried, and the incorporated radioactivity counted. For inhibition assays, fusion protein or rIL-2 at five times the concentration that produced 50% maximal stimulation of CTLL-2 cells was incubated with dilutions of goat anti-IL-2 antibody (30 min, 37°C) prior to addition to the CTLL-2 cells, for assay as described in the previous section.

Peripheral blood lymphocytes were obtained by venepuncture from two healthy donors and prepared by differential centrifugation using Lymphoprep (Nycomed, Oslo, Norway). After washing in RPMI media and seeding into tissue culture plates at \(10^5\) cells/well, appropriate dilutions of fusion protein or rIL-2 were added. Following incubation (36 h, 37°C, 5% CO\(_2\)), 0.5 μCi of \(^3\)H-thymidine was added to each well, and after 4 h of further incubation, cells were harvested and incorporated radioactivity was measured.

FACS Analysis

CTLL-2 cells deprived of IL-2 for 12 h were seeded into Nunc 96-well plates at \(10^5\)/well in 200-μL vol. To the cells was added either a 1:15 dilution of fusion protein, approximating an activity of 100 U IL-2 mL\(^{-1}\), an equivalent dilution of native SCA, rIL-2 at 100 U mL\(^{-1}\), or a mixture of SCA and rIL-2. For competitive inhibition, cells were exposed to 5000 U mL\(^{-1}\) of rIL-2 or TNF at 10 μg mL\(^{-1}\) for 10 min prior to the addition of SCA-IL-2 as above. After incubation, cell-associated SCA epitopes were detected by incubation with rabbit anti-SCA serum and then the bound rabbit anti-SCA antibodies were detected with FITC-conjugated antirabbit IgG (Sigma, St. Louis, MO). After fixing with 1% paraformaldehyde, cell-surface fluorescence was measured using a Becton Dickinson FACScan. Cells were washed five times between steps with RPMI to remove unbound material, and all incubations were for 30 min at 4°C.
RESULTS

Expression and Affinity Chromatography of SCA-IL-2/7 Protein

SCA-IL-2/7 protein expression was induced by addition of 0.1 mM IPTG to transformed cultures of *E. coli* K12 KS476. Figure 2A shows a Western blot of material affinity purified from culture growth medium detected with anti-IL-2 antibody DMS1. This reveals a single band with an apparent mol wt of 46 kDa. When probed with anti-SCA serum, a 46-kDa band was still apparent, but a number of degradation products were revealed (Fig. 2B). Since DMS1 binds to the carboxyl end of IL-2 and does not recognize any of the degradation products, we can assume that proteolysis has removed at least the terminal carboxyl section of the fusion protein in these degradation products.

The ability of SCA-IL-2/7 fusion protein to bind lysozyme is demonstrated in Fig. 3, where affinity-purified material was allowed to bind immobilized lysozyme and was detected with polyclonal anti-IL-2. Furthermore, against a panel of immobilized protein antigens, binding specificity can be demonstrated for lysozyme, but not BSA, KLH, FCS, reconstituted milk, or insulin (Fig. 4). Dose–response curves generated by SCA-IL-2/7 protein in ELISAs with DMS1, which binds the active site of IL-2, are similar to those characteristic of rIL-2 (Fig. 5). For the sample used in these experiments, it would indicate an IL-2 activity of the fusion protein of approx 1200–1500 U·mL⁻¹.

Stimulation of CTLL-2 Cells and Peripheral Blood Lymphocytes by SCA-IL-2/7 Protein

In the conditions employed, half-maximal stimulation of IL-2-dependant CTLL-2 cells occurred at a concentration of approx 0.4 U·mL⁻¹ rIL-2 (Fig. 6). SCA-IL-2/7 protein gave a similar maximal stimulation and dose–response curve. The fusion protein sample achieved a similar half-maximal stimulation at a 5120-fold dilution, giving the activity of the affinity-purified material of approx 1500 U·mL⁻¹ rIL-2 against cells bearing the high-affinity IL-2 receptor. This figure is in close agreement with the estimate derived from the serological assay. Goat anti-rIL-2 sera inhibited the proliferative effects of both rIL-2 and SCA-IL-2/7 protein in a similar manner, giving complete inhibition at 25 mg·mL⁻¹ (Fig. 7).
Fig. 2. (A) Western blot analysis of partially purified fusion protein probed with DMS 1. Lane A: molecular-weight markers, Lane B: rIL-2, Lane C: SCA-IL-2. (B) Fusion protein probed with anti-SCA. Lane D: molecular-weight markers, Lane E: native SCA, Lane F: SCA-IL-2.

The effects of rIL-2 and SCA-IL-2/7 protein on peripheral blood lymphocytes are shown in Fig. 8. The dose-response curve illustrates that the SCA-IL-2 protein acted in a similar manner to IL-2, and the activity of the affinity-purified material corresponds to approx 1600 U·mL⁻¹ rIL-2. This activity against PBLs demonstrates that the SCA-IL-2 is able to stimulate cells bearing the low and intermediate affinity forms of the IL-2 receptor with a similar level of activity, as it has with the high-affinity receptor-bearing cells. Native D1.3 SCA prepared by identical methods had no proliferative action in either assay. (Data not shown.)
ELISA with anti-IL-2

Fig. 3. ELISA with culture supernatants on immobilized lysozyme, probed with anti-IL-2, demonstrating that bound IL-2 epitopes can only be detected in the SCA-IL-2/7 culture supernatant.

FACS Analysis

To determine if the SCA-IL-2 protein is interacting with the cells IL-2 receptors as an intact protein rather than a degraded form consisting of its two parent molecules, FACS analysis was used. The results of the analysis demonstrate that cell-surface-bound SCA epitopes can only be demonstrated in the presence of SCA-IL-2 fusion protein. Figure 9 shows the fluorescence owing to the SCA-IL-2 protein compared with the background sample with no added protein. The FACS results also demonstrate that free native SCA either on its own or in conjunction with rIL-2 does not bind to the cells, with in both cases the detected fluorescence similar to the negative control.

The competitive inhibition of SCA-IL-2-binding by free rIL-2 demonstrates that the fusion protein binds to the cells through the specific receptor–ligand interaction. Competition with an excess of a nonspecific protein (TNF) had no effect on the level of SCA-IL-2 binding. (Data not shown.)
Fig. 4. Binding of SCA-IL-2/7 culture supernatant to a panel of protein antigens. Goat anti-IL-2 sera only detects significant binding of SCA-IL-2/7 to lysozyme.

Results from sequential ELISAs and Western blots also indicate that the fusion protein is stable under the conditions of the biological assays described. (Data not shown.)

DISCUSSION

In this study, we report the construction, expression, and characterization of a fusion protein, SCA-IL-2/7 that has been produced by recombinant DNA methods. The protein retains the antigen-binding characteristics of the parent single-chain antibody and the immunostimulatory actions of IL-2. The construct encodes the pelB leader sequence (20) that directs the expressed protein to the bacterial periplasm, where the oxidizing environment permits the formation of the intramolecular disulfide bonds. Although a considerable amount of the protein was degraded by proteases, a significant quantity of functional material was obtained from the culture supernatant and partially
purified by affinity chromatography. We have demonstrated that the fusion protein retains binding specificity when tested with a panel of antigens and that its immunostimulatory effects can be specifically inhibited by anti-IL-2 antibodies. As tested by serological and biological assays, the fusion protein gave a similar dose–response curve to rIL-2, which we believe demonstrates that the level of activity of the IL-2 in the fusion protein is the same as that of rIL-2. This is in contrast to the activity of the Fab'–IL-2 (8) construct recently described. This apparent difference in activity may be the result of the smaller SCA-IL-2 protein allowing efficient receptor complex internalization, or a steric problem resulting from the joint between Fab' and IL-2 reducing the receptor-binding efficiency. We have demonstrated that the SCA-IL-2 protein is stable under the conditions of the assay, and the FACS results demonstrate that it is intact when it interacts with the IL-2 receptor on the CTLL-2 cell's surface.

This fusion protein interacts with cells bearing either the high- or low-affinity IL-2 receptors. The dual specificity is probably allowed by the relatively long linker between the two parts allowing the amino end of IL-2 the required freedom to interact with the low-affinity receptor. Published work with diptheria toxin-IL-2 fusion proteins has demonstrated the importance of this mobility in allowing this interaction to occur effectively (4,5).

Fig. 5. Detection of IL-2 receptor-binding domains by MAb DMS1 in rIL-2 and SCA-IL-2/7 immobilized on polyclonal anti-IL-2.
CTLL-2 Proliferation Assay

Fig. 6. Stimulatory effects of rIL-2 and SCA-IL-2/7 fusion protein on high-affinity IL-2 receptor bearing CTLL-2 cells. Proliferation is measured by incorporation of $^3$H-thymidine.

Additionally, work in progress demonstrates that the fusion protein is able to cause an increase in vascular permeability in a similar fashion to the effects of IL-2 (21). Although as a systemic effect this can lead to toxicity (22), as a localized event it can enhance the passage of itself and other therapeutic macromolecules into the tumor and ease access for effector cells (23,24).

Although the specificity of the current fusion protein is only appropriate for these preliminary in vitro experiments, recent advances in in vitro selection of antibody variable regions should allow the rapid generation of SCA directed against appropriate cellular targets. In this antibody-guided therapy system, we are targeting the antibodies’ new effector function to the cells of the immune system in the area of the tumor. As a result of this, the requirement for highly selective tumor-associated antigens (TAAs) is reduced, since the accumulation of IL-2 around any inappropriately targeted normal cells will result in little cytolytic action, because effector cells will not interact significantly with normal cells. This contrasts with the potentially detrimental effects of radionuclide or toxin-conjugated antibodies binding to normal cells.
Inhibitory effect of goat anti-IL-2 sera

% inhibition of maximal proliferation

Fig. 7. Inhibition of stimulatory effects of rIL-2 and SCA-IL-2/7 by polyclonal anti-IL-2 antibody. Values are expressed as the percentage inhibition of proliferation when compared with maximally stimulated cells.

PBL Proliferation Assay

Fig. 8. Stimulatory effects of rIL-2 and SCA-IL-2/7 fusion protein on human PBLs. Proliferation is measured by the incorporation of $^3$H-thymidine.
Fig. 9. FACS analysis of binding of SCA-IL-2 fusion protein to IL-2 receptors on the surface of CTLL-2 cells. Binding is demonstrated by the use of the anti-SCA antibody. (A) The level of fluorescence owing to bound SCA-IL-2 is compared to the negative control. (B) SCA-IL-2 is compared to free IL-2. (C) SCA-IL-2 is compared to free IL-2 and free SCA. (D) Competitive inhibition with excess rIL-2 demonstrates a marked reduction in SCA-IL-2 binding. Excess TNF has an excess of an irrelevant cytosis. TNF demonstrates no effect on SCA-IL-2 in binding.
The encouraging preclinical results with rIL-2 have only been partially supported by clinical experience. The poor clinical responses are in part owing to the failure to achieve long-lasting therapeutic concentrations in target tissues and also the systemic toxicity associated with large doses. The targeting of IL-2 by an antibody-derived fusion protein should allow concentration and prolonged action of rIL-2 within the area of the tumor while minimizing systemic toxicity.

The SCA-IL-2 in the form described here will interact with resting PBLs. Therefore, it is unlikely to localize effectively following intravenous administration. However, regional or direct intratumoral administration should result in accumulation, prolonged residence, and an increased antitumor immune response in the tumor. For iv administration and localization, it will be preferable to have a form that interacts only with the high-affinity receptor, as expressed on NK cells and activated T-cells. These cell types are poorly represented in the circulation. Work is in progress to determine if shortening the linker in the SCA-IL-2 construct will produce a protein that will selectively stimulate cells bearing the high-affinity receptor. SCA-IL-2 fusion proteins may provide an effective method of targeting therapeutic doses of rIL-2 to tumors or other targeted cells while significantly reducing systemic toxicity.

REFERENCES

1. Gillis, S., Ferm, M. M., Ou, W., and Smith, K. A. (1978) T cell growth factor: parameters of production and a quantitative microassay for activity. *J. Immunol.* 120, 2027–2032.

2. Rosenberg, S. A., Lotze, M. T., Yang, J. C., Aebersold, P. M., Linehan, W. M., Seipp, C. A., and White, D. E. (1989) Experience with the use of high dose interleukin-2 in the treatment of 652 cancer patients. *Ann. Surg.* 210, 474–485.

3. Fearon, E. R., Pardoll, D. M., Itaya, T., Golumbek, P., Levitsky, H. I., Simons, J. W., Karasuyama, H., Vogelstein, B., and Frost, P. (1990) Interleukin-2 production by tumor cells bypasses T helper function in the generation of an antitumor response. *Cell* 60, 397–403.

4. Williams, D. P., Parker, K., Bacha, P., Bishai, W., Borowski, M., Genbauffe, F., Strom, T. B., and Murphy, J. R. (1987) Diptheria toxin receptor binding domain substitution with interleukin-2: genetic construction and properties of a diphteria toxin-related interleukin-2 fusion protein. *Prot. Engin.* 1, 493–498.
5. Kiyokawa, T., Williams, D. P., Snider, C. E., Strom, T. B., and Murphy J. R. (1991) Protein engineering of diptheria-toxin-related interleukin-2 fusion toxins to increase cytotoxic potency for high affinity IL-2-receptor bearing target cells. *Prot. Engin.* 4, 463--468.

6. Lorderboum-Galski, H., Fitzgerald, D., Chaudary, V., Aldhaya, S., and Pastan, I. (1988) Cytotoxic activity of an interleukin-2-Pseudomonas exotoxin chimeric protein produced in *Escherichia coli.* *Proc. Natl. Acad. Sci. USA* 85, 1922--1926.

7. Landolf, N. F. (1991) A chimeric IL-2/Ig molecule possesses the functional activity of both proteins. *J. Immunol.* 146, 915--919.

8. Fell, H. P., Gayle, M. A., Grosmaire, L., and Ledbetter, J. A. (1991) Genetic construction and characterization of a fusion protein consisting of a chimeric F(ab') with specificity for carcinomas and human IL-2. *J. Immunol.* 146, 2446--2452.

9. Houston, J. S., Levinson D., Mudgett-Hunter, M., Tai, M.-S., Novotny, J., Margolies, M. N., Ridge, R. J., Bruccoleri, R. E., Haber, E., Crea, R., and Opperman, H. (1988) Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli.* *Proc. Natl. Acad. Sci. USA* 85, 5879--5883.

10. Colcher, D., Bird, R., Roselli, M., Hardman, K. D., Johnson, S., Pope, S., Dodd, S., Pantoliano, M. W., Milenic, D. E., and Schlam, J. (1990) In vivo tumor targeting of a recombinant single-chain antigen-binding protein. *JNCI* 82, 1191--1197.

11. Clackson, T., Hoogenboom, H. R., Griffiths, A. D., and Winter, G. (1991) Making antibody fragments using phage display libraries. *Nature* 352, 624--628.

12. Marks, J. D., Hoogenboom, H. R., Bonnett, T. P., McCafferty, J., Griffith, D., and Winter, G. (1991) By-passing immunization. Human antibodies from V-gene libraries displayed on phage. *J. Mol. Biol.* 222, 581--597.

13. McCafferty, J., Griffiths, A. D., Winter, G., and Chiswell, D. J. (1990) Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* 348, 552--554.

14. Stauch, K. L., Johnson, K., and Beckwith, J. (1989) Characterization of degP; a gene required for proteolysis in the cell envelope and essential for growth of *Escherichia coli* at high temperature. *J. Bact.* 171, 2689--2696.

15. Ward, E. S., Gussow, D., Griffiths, A. D., Jones, P. T., and Winter, G. (1989) Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli.* *Nature* 341, 544--546.
16. Smith, K. A., Favata, M. F., and Oroszlan, S. (1983) Production and characterization of monoclonal antibodies to human interleukin 2: strategy and tactics. *J. Immunol.* 131, 1808–1815.

17. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.

18. Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets; procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76, 4350–4354.

19. Gillis, S. and Smith, K. A. (1977) Long term culture of tumour-specific cytotoxic T cells. *Nature* 268, 154–156.

20. Lei, S. P., Lin, H. C., Wang, S. S., Callaway, J., and Wilcox, G. (1987) Characterization of the Erwinia carotovora pelB gene and its product pectate lyase. *J. Bact.* 169, 4379–4383.

21. Savage, P. M., Beynon, H. C., Hartourian, R., Walport, M. J., and Epenetos, A. A. (1992) A single chain antibody interleukin-2 fusion protein mimics the effects of rIL-2 on endothelial cell permeability, in *Cell Adhesion Molecules in Cancer and Inflammation* (Epenetos, A. A., ed.). Chapman and Hall, London.

22. Lotze, M. T., Chang, A. G., Seipp, C. A., Simpson, C., Vetto, J. T., and Rosenberg, S. A. (1986) High dose recombinant interleukin-2 in the treatment of patients with disseminated cancer. *JAMA* 256, 3117–3124.

23. LeBerthon, B., Khawli, L. A., Alauddin, M., Miller, G. K., Charak, B. S., Mazumder, A., and Epstein, A. L. (1991) Enhanced tumor uptake of macromolecules induced by a novel vasoactive interleukin-2 immunoconjugate. *Cancer Res.* 51, 2694–2698.

24. Hennigan, T. W., Begent, R. H. J., and Allen-Mersh, T. G. (1991) Histamine, leukotriene C4 and interleukin-2 increase antibody uptake into a human carcinoma xenograft model. *Br. J. Cancer* 64, 872–874.