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Prolonged and effective blockade of tumor necrosis factor activity through adenovirus-mediated gene transfer

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ABSTRACT **A chimeric protein capable of binding and neutralizing tumor necrosis factor (TNF) and lymphotoxin was expressed in mice transduced with a replication-incompetent adenoviral vector into which a TNF inhibitor gene had been engineered. Within 3 days following the injection of 10^9 infectious particles, the TNF inhibitor concentration exceeded 1 mg/ml of plasma; this level of expression was maintained for at least 4 weeks, and detectable TNF inhibitory activity was measured 6 weeks after injection of the recombinant virus. Introduction of the artificial gene produced a phenotypic effect comparable to homozygous deletion of the 55-kDa TNF receptor, in that animals were rendered highly susceptible to infection by *Listeria monocytogenes*, whereas control animals receiving a replication-incompetent virus coding for β-galactosidase were capable of resisting *Listeria* challenge. Adenovirus-mediated transfer of a gene encoding a TNF inhibitor offers a practical means of imposing effective, long-term blockade of TNF activity *in vivo* for investigational and therapeutic purposes.**

The analysis of cytokine function, and the treatment of cytokine-mediated disease states, have depended upon the development of techniques for long-term blockade of cytokine activity. Passive immunization has been used with success as a means of neutralizing tumor necrosis factor (TNF) activity in animal models of sepsis (1–6), cancer (7), and chronic bacterial infection (8, 9). Such studies have revealed that TNF is important to host defense against *Listeria monocytogenes* (9), *Mycobacterium bovis* (8), and *Cryptococcus neoformans* (10), each of which replicates intracellularly.

However, passive immunization has several shortcomings as a means of blocking TNF activity over long periods of time. Among these, specific antibodies must be produced for each animal species in which they are used; the antibodies serve as immunogens and can only be administered for a limited period of time; and most antibodies raised against TNF are incapable of neutralizing lymphotoxin, a related cytokine with an identical spectrum of biological activities transduced through binding to the same set of receptors as those engaged by TNF (11–13). Moreover, the neutralizing potential and other properties of antibodies are quite variable, and conclusions drawn from passive immunization studies cannot always be accepted as general and correct.

Deletion of the TNF receptor genes has been achieved (refs. 14 and 15; D. Goeddel, personal communication) and may provide a means to study the function that TNF evolved to serve in mice. However, studies of TNF action in specialized strains of mice and in other mammalian species have yet to be advanced by this achievement. Toward the development of an alternative and versatile means of selectively blocking the action of molecules that act through interaction with the two known TNF receptors, we recently produced a recombinant protein capable of binding TNF and lymphotoxin and neutralizing their activity. This molecule, a fusion protein formed by joining the human 55-kDa TNF receptor extracellular domain to a mouse IgG heavy chain, binds TNF at a 1:1 molar ratio, engaging two of its three receptor-binding sites. In an effort to permit flexible, long-term neutralization of TNF in animals of diverse species, we developed an adenoviral vector encoding the chimeric TNF inhibitor, transcriptionally driven by a cytomegalovirus (CMV) promoter.

MATERIALS AND METHODS

Generation of Recombinant Adenoviruses. The bivalent TNF inhibitor cDNA (16) was subcloned (*Eco*RI → *Sal* I) into pACCMV (obtained from Robert Gerard, University of Texas Southwestern Medical Center), which contains (sequentially) 1.3 map units of sequence taken from the left end of the adenovirus 5 (Ad5) genome, the CMV early promoter, the pUC19 polylinker, simian virus 40 splice and poly(A) signal sequences, and, finally, map units 9 through 17 of the Ad5 genome. The recombinant plasmid was cotransfected into the 293 cell packaging line together with the large adenoviral plasmid pJM17 (obtained from Frank L. Graham McMaster University, Hamilton, Ontario, Canada) using a calcium phosphate coprecipitation method. As described by Graham and Prevec (16), 293 cells constitutively express the adenoviral E1A and E1B proteins and support the replication of E1A-defective mutants. pJM17 supplies the remainder of the Ad5 genome, but its size exceeds the packaging limit for adenovirus. Adenoviral genomes formed by recombination between the pJM17 vector and the pACCMV vector contained the inhibitor expression construct, were replication defective, and were effectively packaged to form infectious virions.

After cotransfection, cells were overlaid with Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% fetal bovine serum (FBS) (both from GIBCO), and 0.65% Noble agar (Difco). Plaques were picked 10–14 days after cotransfection and propagated in 60-mm plates of 293 cells. Viral DNA was extracted (16) from the propagated plaques and screened by Southern blotting. Conditioned medium was screened for TNF inhibitory activity (i.e., neutralization of recombinant murine TNF by the SKMEL-109 bioassay system) (17).

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Abbreviations: TNF, tumor necrosis factor; CMV, cytomegalovirus; FBS, fetal bovine serum; CHO, Chinese hamster ovary; pfu, plaque-forming units; cfu, colony-forming units; Ad5, adenovirus 5.

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A second recombinant adenovirus, encoding β-galactosidase, was made by subcloning the *lacZ* gene (from pNβ; kindly provided by Grant MacGregor, Howard Hughes Medical Institute, Houston) (18) into pACCMV and cotransfecting with pJM17 as outlined above. Candidate plaques were screened by infecting 293 cells, fixing with 4% paraformaldehyde in phosphate-buffered saline (PBS), and staining for β-galactosidase activity using a solution of 10 mM $K_4Fe(CN)_6$, 10 mM $K_4Fe_3(CN)_6$, 2 mM $MgCl_2$, (all from Sigma) and 5-bromo-4-chloro-3-indolyl β-D-galactoside (United States Biochemical) at a concentration of 1 mg/ml in Dulbecco's PBS.

Propagation and Purification of Recombinant Adenoviruses. 293 cells were grown in DMEM/F12 medium supplemented with 5% FBS and 2% penicillin/streptomycin solution (GIBCO) in 15-cm plates or roller bottles and infected at a multiplicity of 5–10. After 36–48 hr, when the cytopathic effect was complete, the cells were sedimented at 500 × *g*. They were then resuspended in 10 ml of DMEM/F12 medium, and the virus was released by freeze-thawing five times. It was then purified by ultracentrifugation over a CsCl gradient (16). Banded virus was recovered and spin-dialyzed over Sepharose CL6B (Pharmacia) and stored in aliquots at −80°C after the addition of bovine serum albumin to a concentration of 100 μg/ml. To titer the final preparation, an aliquot of virus was serially diluted and assayed for ability to form plaques on 293 cell monolayers (16).

Assay of TNF Inhibitory Activity. Attempts to directly demonstrate the TNF inhibitor in plasma by polyacrylamide gel electrophoresis were unsuccessful, probably because the inhibitor is glycosylated and therefore migrates as a broad band, overlapping with many other plasma proteins (17). Therefore, TNF inhibitory levels were measured in cell culture medium and in mouse plasma as described (17). Briefly, samples were serially diluted and 1 μl of each dilution was incubated in separate wells of a 96-well plate with murine TNF at a final concentration of 1.0 ng/ml. The incubation was allowed to continue for 1 hr at 37°C in a final volume of 150 μl, in the presence of cycloheximide at a concentration of 100 μg/ml. Seventy thousand SKMEL-109 cells were then added to each well and the incubation was continued overnight. The plates were then washed twice in 0.9% NaCl and stained with crystal violet (0.5% in 30% methanol) for 5 min. The plates were then washed four more times. Cells that had survived TNF treatment were quantitated by solubilizing the stain in 50% acetic acid. Optical density was determined at 490 nm. One neutralizing unit was defined as the quantity of inhibitor required to neutralize 150 pg of recombinant mouse TNF. *Minimum* estimates of plasma inhibitor concentrations were calculated with reference to the concentration of plasma required to effect complete inhibition of TNF activity—i.e., complete neutralization of TNF by a given concentration of plasma inhibitor reflected the presence of at least an equimolar concentration of active inhibitor. Given the affinity constant of the inhibitor/ligand couple (15, 18, 19), and the initial concentration of TNF in the assay system (1 ng/ml), the approximation is a reasonably close one.

Iodination of Chimeric Inhibitor. The TNF inhibitor was purified from conditioned medium of Chinese hamster ovary (CHO) cells permanently transfected with the inhibitor expression construct (17). This was accomplished by NH_4SO_4 precipitation followed by protein G and protein A chromatography (both from Pharmacia). Although the inhibitor failed to bind to protein G-Sepharose, it bound avidly to protein A-Sepharose in the presence of 3 M NaCl and was eluted using 0.01 M acetic acid and immediately neutralized using 0.05 M bicine (pH 8.8). Purified inhibitor was then iodinated using the Iodo-Gen technique (20). Radiolabeled inhibitor was separated from unincorporated ^{125}I by chromatography over Bio-Gel P-6 (Bio-Rad) to yield a product with a specific activity of 1.5×10^3 cpm/ng of inhibitor.

Pharmacokinetic Studies. Labeled inhibitor protein was injected (5×10^5 cpm per mouse) via the orbital sinus, and heparinized plasma was obtained at 5 min and at 1, 3, 6, 24, 60, and 72 hr after injection by bleeding from the tail. Three microliters of plasma obtained at each time point was electrophoresed through a 10% SDS/polyacrylamide gel (21). The gel was dried, exposed to a Europium screen for 24 hr, and scanned using a PhosphorImager (Molecular Dynamics). The radiolabeled protein in circulation was thus quantitated, and calculations of the volume of distribution and half-life were made assuming a two-compartment model (22).

***L. monocytogenes* Infection.** *L. monocytogenes* organisms (ATCC no. 43251; obtained from Michael Bevin, Howard Hughes Medical Institute, Seattle) were stored at −70°C at an approximate titer of 2×10^8 colony-forming units (cfu)/ml. Prior to administration, mice received 10^9 plaque-forming units (pfu) of either adenovirus engineered to express β-galactosidase (control) or adenovirus engineered to express the TNF inhibitor. Forty-eight hours after inoculation of the viruses, mice were bled to confirm that high concentrations of TNF inhibitor had been achieved in plasma. Seventy-two hours after inoculation, the bacteria were thawed and 2.4×10^4 organisms ($LD_{50} = 10^5$ cfu in C57B/6 mice; Michael Bevan, personal communication) were administered by orbital sinus injection. The titer of the bacterial inoculum was confirmed retrospectively by plating serial dilutions on tryptic soy agar (Difco). Four days after administration, animals were sacrificed by CO_2 narcosis. The liver and spleen from each animal were weighed and homogenized in PBS. Serial dilutions of the organ homogenates were plated on tryptic soy agar to determine cfu of *Listeria* per g of tissue.

Animals. Mice of the strains BALB/c (for pharmacokinetic studies) and C57B/6 (for studies with *Listeria* infection) were obtained from Sasco and from Harlan, respectively, and housed in the University of Texas Southwestern Medical Center Animal Resource Center prior to the experiments shown. All studies were carried out in accordance with requirements of the institutional review board for animal experimentation.

RESULTS

Pharmacokinetics of the TNF Inhibitor Produced by the Adenoviral Construct. BALB/c mice were injected with 10^9, 10^8, or 10^7 pfu of the TNF inhibitor virus. Plasma inhibitory activity, which was undetectable in samples obtained prior to injection of the adenovirus, rose abruptly, peaking on day 2 in mice that received 10^7 pfu and on day 8 in mice that received 10^8 or 10^9 pfu (Fig. 1*A*). Between dosage groups, peak plasma concentrations were separated by approximately one order of magnitude, reflecting the dose dependency of inhibitor production. Control mice that received 10^9 pfu of virus encoding β-galactosidase had no detectable plasma inhibitor activity at any time point (data not shown). Approximately 10% of the hepatocytes of the control mice were transduced by this dose of virus, as indicated by staining for β-galactosidase activity (Fig. 1*B*).

A rapid decline of inhibitor production was observed by day 10 in the group of mice inoculated with 10^8 pfu and by day 28–35 in the group of mice inoculated with 10^9 pfu. Since the protein encoded by the adenovirus contained a human receptor moiety, we considered it possible that antibody formation eventuated in rapid clearance of the recombinant protein. To investigate whether this was so, we injected mice from the group that had received 10^9 pfu and had ceased to express inhibitory activity in the plasma for a period of 4 weeks, as well as control animals that had not been exposed to the recombinant protein, with purified inhibitor labeled with ^{125}I and analyzed the distribution

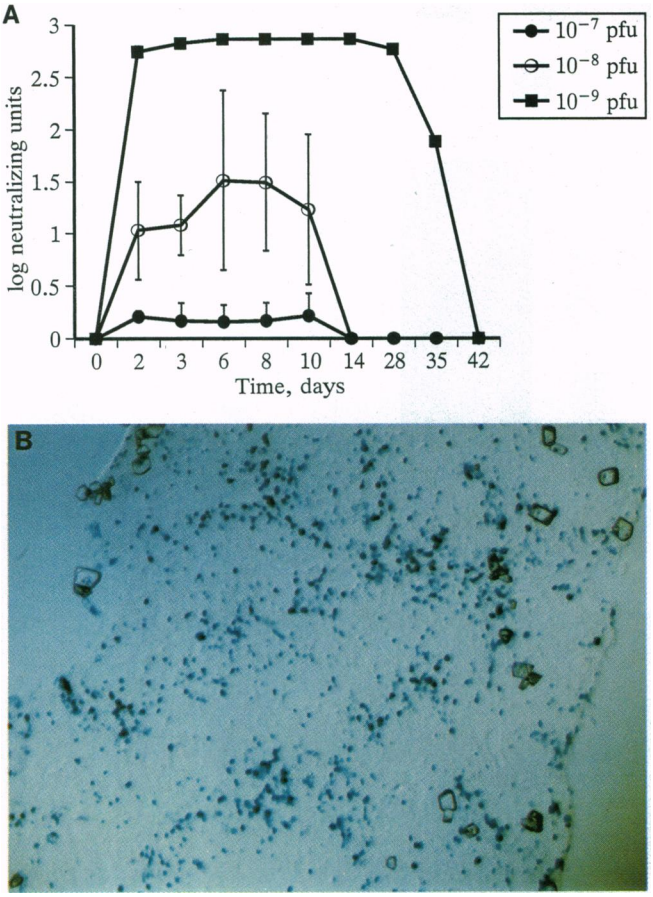

FIG. 1. (*A*) Graphic representation of inhibitor activity in the plasma of mice following infusion of the inhibitor virus at the doses indicated. Note that inhibitory activity is presented according to a logarithmic scale and that the time axis is nonlinear after 14 days. Mice that were not injected with the inhibitor virus (not shown) have no detectable TNF inhibitory activity in plasma. Data represent average inhibitory activity in plasma sampled from three mice. Standard deviation is indicated by error bars (error was too small to print in the group that received 10^9 pfu). (*B*) β-Galactosidase activity identified by histochemical staining of the liver in mice that received 10^9 pfu of an adenovirus encoding the *Escherichia coli lacZ* product by an intravenous route. Approximately 10% of the hepatocytes have been transduced. (×50.)

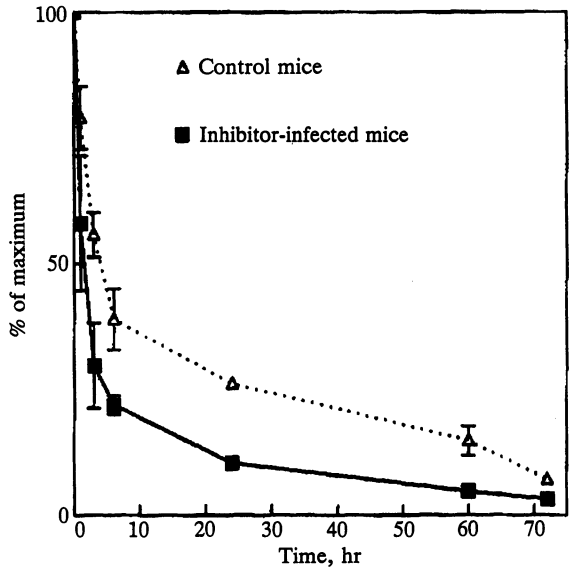

FIG. 2. Pharmacokinetics of inhibitor clearance in mice that have previously been inoculated with adenovirus encoding the TNF inhibitor and in control mice that have not been exposed to adenovirus. Radiolabeled inhibitor was injected at t = 0. Mice (three per group) were bled repeatedly after the intervals of time noted, and inhibitor remaining in the plasma was quantitated (see text). The quantity of radioactivity present in the plasma after 5 min was taken to be maximal, and other values were expressed as a percentage of this figure. Error bars indicate standard deviation among measurements. Redistribution was calculated based on the first four points, and half-life was estimated based on the last four points of each curve.

and clearance kinetics of the protein. In both groups of animals, inhibitor clearance was consistent with a two-compartment model, in which a rapid distribution phase was followed by a first-order elimination phase (Fig. 2). In mice previously inoculated with the inhibitor virus, the volume of distribution was 5 ± 1.5 ml (mean ± SD) and the half-life was 29 ± 0.1 hr (mean ± SD). In control control mice, the volume of distribution was 6.8 ± 1.5 ml, and the half-life was 36 ± 6.8 hr. The difference in half-life was thus small and beneath statistical significance ($P > 0.05$, Student's *t* test). Therefore, we could not explain the fall in inhibitor levels on the basis of shortened half-life resulting from immunization and presume that production of the inhibitor declines several weeks following viral transduction. Seventy-two hours after infection, similar inhibitor concentrations were observed in C57B/10 mice and in BALB/c mice (662 ± 36.6 and 739 ± 115 neutralizing units/μl, respectively). Therefore, the adenoviral vector is expressed equally well in two different strains of *Mus musculus*.

Administration of the TNF Inhibitor Virus Predisposes to Listeriosis. C57B/6 mice were challenged with 2.4×10^4 cfu of *Listeria* organisms by an intravenous route 72 hr after receiving either 10^9 pfu of the TNF inhibitor virus or 10^9 pfu of the β-galactosidase virus. Biological assays revealed that all animals receiving the inhibitor virus had high inhibitory activity in their plasma (corresponding to ≈1 mg of inhibitor per ml) by this time following inoculation. Twenty-four hours after receiving the *Listeria* injection, both groups of mice had developed piloerection and appeared clinically ill. However the mice given the inhibitor virus continued to deteriorate, experiencing an average weight loss of 3.5 g per animal within 72 hr. There was no significant weight loss in the control group. The animals were sacrificed on day 4, by which time those animals that had received the inhibitor virus appeared moribund. The liver and spleen were homogenized in PBS to determine the *Listeria* burden. There was a highly significant (between 10,000- and 100,000-fold) difference between *Listeria* counts in the liver and spleen of animals pretreated with the TNF inhibitor virus and those pretreated with the control virus (Fig. 3*A*), and gross infection of the liver and spleen of animals treated with the inhibitor virus was apparent at necropsy (Fig. 3*B*). Thus, infection with the inhibitor virus yields a phenotype similar to passive immunization against TNF (9) and to homozygous deletion of the 55-kDa TNF receptor (14, 15).

DISCUSSION

The adenovirus-mediated transfer of a TNF inhibitor gene represents a means for imposing blockade of TNF activity in virtually any mammalian species and maintaining it for a period of weeks to months, or possibly longer. Conceptually, the approach that we have used differs from passive immunization or the infusion of recombinant inhibitory proteins, in that the function of the TNF gene has been abrogated by introduction of a second, artificial gene, the product of which neutralizes TNF. In effect, the TNF inhibitor gene creates a dominant negative phenotype, manifested when only a fraction of the cells of the host have been transduced. The TNF inhibitor gene, transduced alone or in conjunction with other,

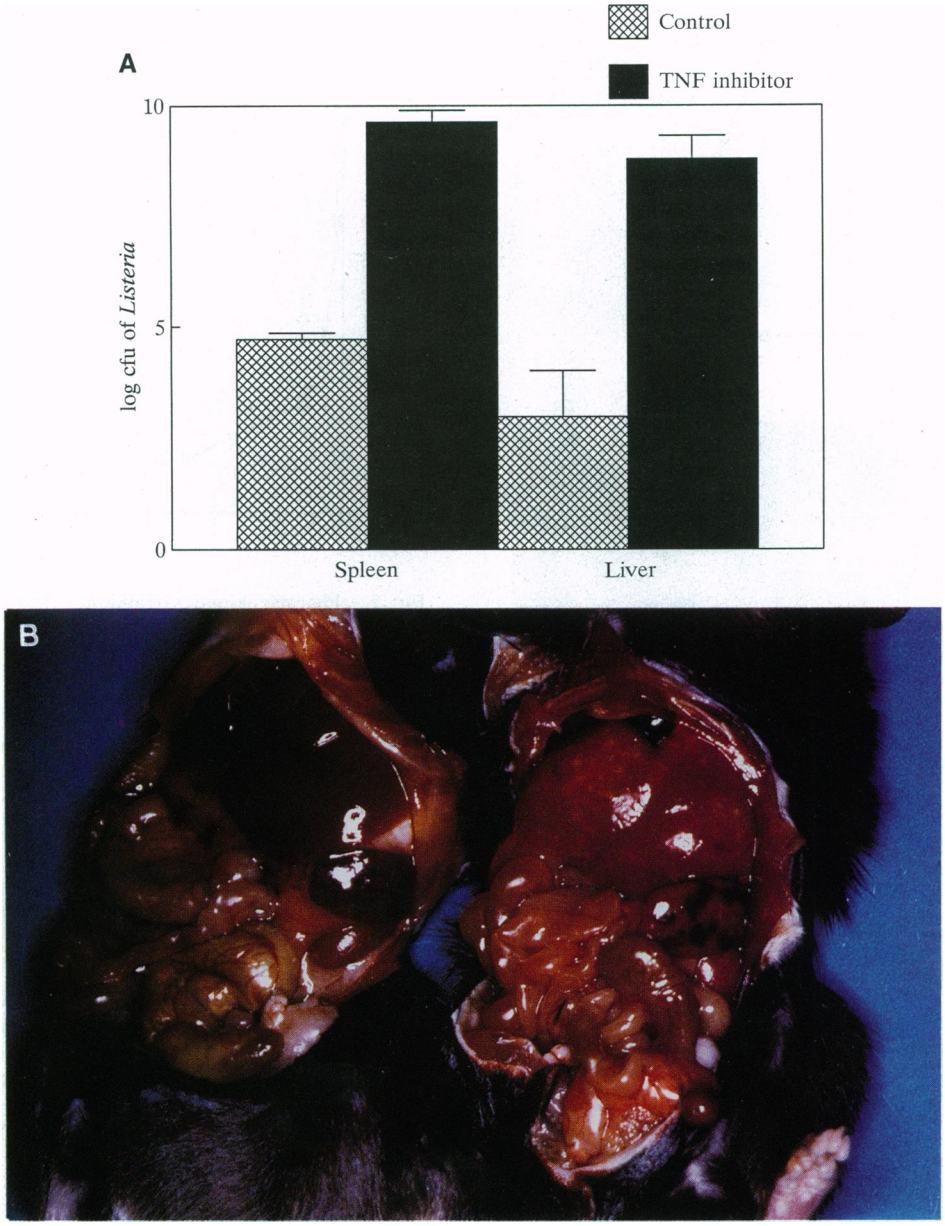

FIG. 3. (*A*) Appearance of the liver and spleen of representative mice inoculated with 4×10^3 cfu of *L. monocytogenes* 3 days after receiving 10^9 pfu of adenovirus coding for β-galactosidase (control) or TNF inhibitor. Note that the liver and spleen are of normal appearance in the control animal and are whitish in color in animals that received the inhibitor adenovirus. (*B*) cfu of *L. monocytogenes* present in the liver and spleen of animals pretreated with the control adenovirus or the TNF inhibitor adenovirus. Error bars represent standard deviation among three animals treated with each virus. (×100.)

similar inhibitor genes, presents a method for analysis of essential cytokine functions.

The TNF inhibitor, here expressed in mice, is a chimeric protein consisting of a human TNF receptor extracellular domain fused to a mouse IgG heavy chain (17). Though the formation of antibodies against this molecule might be anticipated, it did not occur to an extent sufficient to shorten the half-life of the molecule in circulation nor to an extent sufficient to allow detection by immunoprecipitation of radiolabeled inhibitor *in vitro* (not shown). This may reflect low intrinsic immunogenicity of the 55-kDa receptor extracellular domain, though the human and murine receptors differ by ≈40% at the amino acid level. Alternatively, the failure to develop antibodies might reflect the importance of TNF in the generation of a humoral immune response (23). The use of the adenoviral transduction system therefore seems to offer a practical approach to the production of large quantities of the inhibitor *in vivo*, over a long period of time.

The quantity of inhibitor synthesized may be estimated from its steady-state concentration, its half-life, and its volume of distribution *in vivo*. The half-life of inhibitor activity *in vivo* was found to be ≈1 day, and the apparent volume of distribution, calculated from the average redistribution that occurs following intravenous injection of radiolabeled inhibitor protein, is 5.9 ± 1.5 ml. This suggests that 3 days following infection, 3 mg of inhibitor is synthesized daily in the tissues of mice transduced by infusion of 10^9 infectious units. The infusion of 10^9 pfu of an adenovirus encoding β-galactosidase leads to transduction of ≈10% of the hepatocyte population. This, together with the linear relationship between peak inhibitor level and viral dose, suggests that a point of saturation has not been approached with the administration of 10^9 pfu. Since 10^{11} to 10^{12} infectious units of recombinant virus can be produced in the laboratory without difficulty, manufacture of gram quantities of the

inhibitor *in vivo* on a daily basis might easily be attained using mice or larger animals. This quantity of inhibitor could not easily be produced *ex vivo*, given that the protein can only be expressed in eukaryotic cells (B.B. and K.P., unpublished observation). Therefore, the use of an adenoviral gene transfer system for production of the inhibitor represents a major technical advance.

The primary cellular target of the adenoviral vector is the hepatocyte, although endothelial, muscle, lung, spleen, and mesothelial cells are also readily infected (16, 24–26). The decline in inhibitor biosynthesis that is observed after several weeks might reflect dilution of the transferred gene, which remains episomal and does not undergo replication in the host, or shutoff of the CMV expression apparatus mediated by the host cell. From the studies performed to date, we are unable to determine which of these mechanisms explains the decline in expression or whether both play a contributory role. However, for practical purposes, the period of expression is quite sufficient to allow investigation of TNF function.

Based upon a conservative estimate of the affinity constant of the bivalent inhibitor [$K_a = 10^{11}$ M (17, 27)], expression of the inhibitor protein at a concentration of 1 mg/ml in plasma should lead, at equilibrium, to the engagement of >99.9998% of TNF molecules that are present within this compartment. Thus, high-level expression of the TNF inhibitor functionally mimics deletion of genes coding for proteins capable of engaging the 55-kDa TNF receptor (e.g., TNF and the lymphotoxins). Although it cannot immediately be assumed that all fluid compartments are equivalent in their content of binding protein, mice injected with the inhibitor virus become demonstrably immunodeficient, as suggested by their high susceptibility to infection by *L. monocytogenes*. Thus, the inhibitory activity measured in plasma correlates with an altered phenotype *in vivo*, which represents a precise phenocopy of homozygous 55-kDa TNF receptor deletion (14, 15). The affinity of the 75-kDa TNF receptor for TNF and for lymphotoxin is similar to the affinity of the 55-kDa receptor (27). Since the recombinant adenovirus produces a phenocopy of 55-kDa receptor deletion, and since the inhibitor denies the 75-kDa receptor and the 55-kDa receptor access to TNF or lymphotoxin, it may reasonably be considered that administration of the inhibitor mimics a double deletion—i.e., deletion of the 55-kDa and 75-kDa receptors or deletion of all of the TNF loci. It may therefore be anticipated that, for many applications, introduction of the inhibitor gene constitutes a reasonable alternative to double deletion of the TNF or TNF receptor genes and has the additional advantage of being akin to a "conditional knockout." Moreover, our data suggest that deletion of the TNF loci and deletion of the TNF receptor loci will yield phenotypically identical results, in contrast to the phenotypic discrepancies that result from deletion of certain hormones and their receptors (e.g., the interleukin 2 protein and its receptor) (28, 29).

For some time, questions have been raised concerning the action of TNF in chronic autoimmune states (30–32), such as systemic lupus erythematosus [modeled by the NZW/NZB F_1 hybrid mouse (33–35)] and type 1 diabetes [modeled by mice of the nonobese diabetic (NOD) strain (36)]. Whereas passive immunization of such animals might not be practical given the long latent period of the disease, the use of the TNF inhibitor expression system described here will allow an assessment of the importance of this and other cytokines in the pathogenesis of autoimmunity. Moreover, the cooperative effects of different members of the TNF family of genes and their receptors (e.g., the 55-kDa receptor and the Fas antigen) may be assessed by administration of the TNF inhibitor adenovirus (or analogous inhibitor adenoviruses) to animals bearing preexisting mutations in homozygous form. Finally, to the extent that overproduction of cytokines contributes to the development of human diseases, virally encoded cytokine inhibitors may ultimately be applied in a form of gene therapy.

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