

GLUCOCORTICOID-MEDIATED INHIBITION OF
ENDOTOXIN-INDUCED INTRATUMOR TUMOR NECROSIS
FACTOR PRODUCTION AND TUMOR HEMORRHAGIC
NECROSIS AND REGRESSION

BY ROBERT J. NORTH AND EDWARD A. HAVELL

From The Trudeau Institute, Saranac Lake, New York 12983

It is well documented (1) that parental injection of bacterial endotoxin results in extensive hemorrhagic necrosis of most solid murine tumors, but in complete regression of only some. There is evidence (2) that the tumors that undergo complete regression are those that are of sufficient immunogenicity, and that regression, as opposed to hemorrhagic necrosis, is immunologically mediated. In support of this interpretation are published results showing (3) that, in the case of at least one immunogenic murine tumor, endotoxin-induced regression depends on the acquisition by the host of a sufficient number of tumor-sensitized CD4⁺ T cells at the time that endotoxin is given. It was suggested (4), on the basis of this and other evidence, that the therapeutic action of endotoxin depends on its ability to induce an intratumor hemorrhagic reaction that is destructive enough to reduce the tumor to a size that host concomitant immunity is capable of rejecting.

Most evidence favors the view that endotoxin-induced tumor hemorrhagic necrosis is mediated by TNF. It is known, for example, that natural TNF from appropriate endotoxin-treated donor mice, and rTNF, can substitute for endotoxin in causing tumor hemorrhagic necrosis (5-8). Again, endotoxin-induced tumor hemorrhagic necrosis has been shown to be associated with the intratumor synthesis of TNF (4), and neutralization of this TNF by treating the host with an anti-TNF antibody can partially inhibit tumor hemorrhagic necrosis (4).

Because macrophages are the predominant producers of TNF, and because these cells are highly represented in tumors (9), it is likely that they are the principle source of intratumor TNF (10, 11). However, the ability of macrophages to make TNF in vitro can be blocked by treating them with glucocorticoids (12). It was anticipated, therefore, that glucocorticoid treatment would also block endotoxin-induced intratumor TNF production in vivo and consequently tumor hemorrhagic necrosis. The purpose of this paper is to show that this is the case. It will also show that, whereas glucocorticoid treatment inhibits the ability of endotoxin to cause tumor hemorrhagic necrosis and regression, it has no inhibitory effect on tumor hemorrhagic necrosis and regression caused by exogenous TNF.

This work was supported by grants CA-16642 and CA-27994 from the National Cancer Institute and by a grant from the Oliver S. and Jennie R. Donaldson Trust.

Materials and Methods

Mice. AB6F₁ (A/J × C57BL/6) female mice were used when they were 10-12 wk old. They were purchased from The Trudeau Institute Animal Breeding Facility, and were free of common viral pathogens according to the results of tests routinely performed by Charles River Professional Services, Wilmington, MA.

Tumor. The SA1 spindle cell sarcoma syngeneic in A/J mice was obtained some years ago from The Jackson Laboratories, Bar Harbor, ME. It was passaged as an ascites and prepared for initiating tumors as described previously (13). Tumors were initiated intradermally in the abdominal region by implanting 10⁶ tumor cells in 0.05 ml of PBS. Tumor growth and regression was followed by monitoring changes in the mean of two diameters measured at right angles with dial calipers.

Endotoxin and TNF. Murine rTNF was a gift from Dr. Jan Tavernier of the University of Ghent, Belgium. It has a specific activity of 1.37 × 10⁸ U/mg protein, and contained <10 ng endotoxin/mg (13). Endotoxin (*Salmonella enteritidis*, batch 3105-25) was obtained from Difco Laboratories, Detroit, MI. Both reagents were dissolved at appropriate concentrations in PBS. They were injected in a volume of 0.2 ml in a lateral tail vein on day 9 of tumor growth. The dose of rTNF was 10⁵ U, and the dose of endotoxin 25 μg.

Quantifying the Tumor Hemorrhagic Reaction. The rate and extent of TNF- or endotoxin-induced intratumor hemorrhaging were quantified by measuring the accumulation in the tumor of ⁵¹Cr-labeled syngeneic RBCs infused 1 h earlier in a lateral tail vein (13). Briefly, AB6F₁ mice were bled by cardiac puncture into a syringe containing citric acid/dextrose solution. The RBCs were pelleted and washed by centrifugation in RPMI 1640 medium. To each 0.5 ml of packed red cells was added 0.5 ml of RPMI 1640 medium containing 100 μCi of Na₂⁵¹CrO₄ having a specific activity of 250-500 mCi/mg (Amersham Corp., Arlington Heights, IL). The RBCs were suspended and incubated for 1 h at 37°C, washed three times, and resuspended in 2 ml of PBS for intravenous infusion. Each mouse was given an infusion of 0.2 ml of the ⁵¹Cr-labeled RBC suspension in a lateral tail vein 1 h before treatment with endotoxin or rTNF. To measure intratumor hemorrhaging five mice were killed at 1, 2, 4, and 6 h after injection of endotoxin or rTNF, their tumors were excised, placed in glass vials, and the ⁵¹Cr content per tumor determined with a Rack Gamma II Counter (LKB Instruments, Inc., Gaithersburg, MD). The results are presented as means of the total counts per minute per tumor.

Assay for TNF. An assay based on the procedure of Wang et al. (14) was used. Briefly, 1.5 × 10⁴ murine L929B cells in 100 μl of Eagle's MEM (Microbiological Associates, Walkersville, MD) containing 5% FCS and antibiotics were placed in wells of 96-well microtiter plates and incubated overnight at 37°C in a 5% CO₂ humidified incubator. Sequential twofold dilutions of the TNF-containing extracts were made in the same medium containing 2 μg of actinomycin D (Calbiochem-Behring Corp., La Jolla, CA), and 100 μl of each dilution added to replicate wells containing the L929B target cells. After 24 h of incubation cytotoxicity was scored microscopically. The cytotoxicity titer (U/ml) is defined as the highest dilution of the test material capable of causing 50% or more destruction of the monolayer of L929B cells. The assay was used to determine cytotoxic titers of extracts of tumors from control and endotoxin-treated mice. Extracts were made by homogenizing whole tumors (10% wt/vol) in PBS (pH 7.4) containing 10% FCS. Homogenization was done on ice with a motorized blender. The homogenates were centrifuged to pellet tissue debris, and the clarified supernatants assayed as above. Previous studies have shown (4) that TNF is stable during the extraction procedure, and that all endotoxin-induced cytotoxic activity in tumor extracts is neutralizable with an anti-rTNF antibody.

Glucocorticoids. Cortisone acetate suspended in saline (Merck, Sharp and Dohme, West Point, PA) and dexamethasone 21-acetate (Sigma Chemical Co., St. Louis, MO) dissolved in ethanol and then in PBS were injected in a volume of 0.2 ml subcutaneously in the nape of the neck in a dose of 2 or 1 mg, respectively.

Results

Glucocorticoid Treatment Inhibits Endotoxin-induced Hemorrhagic Necrosis and Regression. The first experiments were designed to investigate whether intradermal in-

jection of 2 mg of cortisone acetate would prevent a 25- μ g dose of endotoxin given intravenously 12 h later from causing hemorrhagic necrosis and regression of a 9-d SA1 sarcoma. As can be seen in Fig. 1, whereas endotoxin treatment caused an extensive hemorrhagic reaction in and subsequent regression of the SA1 sarcoma growing in control mice, it caused neither hemorrhagic necrosis nor complete regression of the same sized tumor in glucocorticoid-treated mice. There was a small degree of endotoxin-induced regression of the tumor in cortisone-treated mice, but progressive tumor growth soon resumed.

Fig. 2 shows the results of an experiment designed to determine whether dexamethasone was also capable of inhibiting endotoxin-induced hemorrhagic necrosis. It can be seen that this glucocorticoid given 12 h before endotoxin was as effective as cortisone acetate in inhibiting hemorrhagic necrosis. Dexamethasone was also as capable of inhibiting tumor regression (results not shown).

Glucocorticoid Treatment Inhibits Intratumor TNF Production. Table I shows the results of an experiment that measured the ability of cortisone acetate or dexamethasone to inhibit endotoxin-induced synthesis of TNF in a 9-d SA1 sarcoma and in the host at large. This experiment involved measuring the capacity of serial dilutions

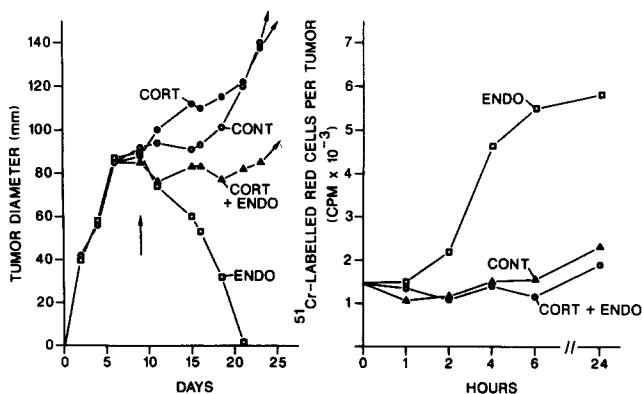


FIGURE 1. The ability of a 25- μ g i.v. dose of endotoxin (*ENDO*) given on day 9 of tumor growth (*arrow*) to cause intratumor hemorrhaging (*right panel*) and tumor regression (*left panel*) was severely inhibited by an intradermal injection of cortisone acetate 12 h before endotoxin (*CORT + ENDO*). Cortisone acetate alone had little effect on tumor growth. Means of five mice per group.

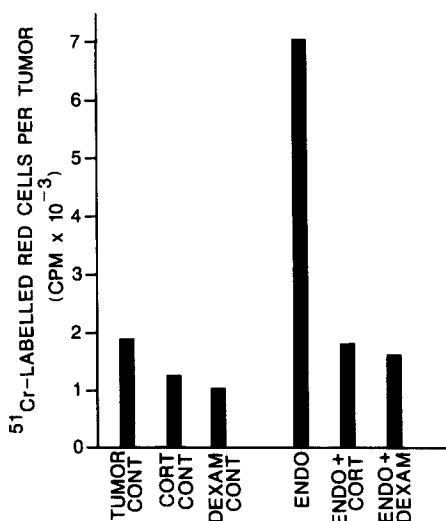


FIGURE 2. Dexamethasone (*ENDO + DEXAM*) was as effective as cortisone acetate (*ENDO + CORT*) at inhibiting intratumor hemorrhaging caused by endotoxin (*ENDO*) given on day 9 of tumor growth. The tumor hemorrhagic reaction was measured at its peak 6 h after giving endotoxin. Means of five tumors per group.

TABLE I
Effect of Glucocorticoids on Endotoxin-induced TNF Production

Group	TNF activity (U/organ)		
	Tumor	Spleen	Serum
Control	BDL*	BDL	<16
Endotoxin	4,642	853	512
Cortisone acetate & endotoxin	299	128	<16
Dexamethasone & endotoxin	299	64	<16

On day 9 of SA1 sarcoma growth mice received 2.5 mg of cortisone acetate or 1 mg of dexamethasone sc 12 h before giving 24 μ g i.v. of endotoxin. Homogenates (10% wt/vol) of five pooled tumors or spleens were prepared. Homogenate supernatants and pooled sera were assayed for TNF. TNF/organ = (homogenate vol \times TNF U/ml)/5. Total serum TNF assumes 1 ml serum/mouse.

* TNF activity below detectable levels (BDL) at a 1/16 dilution of supernatant from a 10% wt/vol homogenate.

of extracts of tumors and organs from glucocorticoid-treated and control mice to lyse L929B target cells in vitro. A previous study showed (4) that all of the cytotoxicity extractable from the tumors and organs of endotoxin-treated mice is due to the presence of TNF, and that peak quantities of TNF are synthesized 2 h after endotoxin is given.

It can be seen that 2 h after endotoxin treatment appreciable quantities of TNF were present in the tumors, spleens, and blood of endotoxin-treated control mice, and that by far the largest quantity of TNF was present in the tumors. In contrast, greatly reduced quantities (10% or less of controls) of TNF were present in the tumors, spleen, and blood of endotoxin-treated mice pretreated with glucocorticoids.

Glucocorticoid Treatment Has No Effect on the Ability of Exogenous TNF to Cause Tumor Hemorrhagic Necrosis and Regression. It is well documented that intravenously injected natural or rTNF results in extensive hemorrhagic necrosis of the centers of murine tumors, and that in the case of the SA1 sarcoma, hemorrhagic necrosis is followed by complete tumor regression (4). Given the published evidence that TNF is the mediator of endotoxin-induced regression (4), and the evidence above that glucocorticoids inhibit endotoxin-induced hemorrhagic necrosis by inhibiting endogenous TNF synthesis, it was anticipated that glucocorticoid treatment would have no effect on the ability of exogenous TNF to cause hemorrhagic necrosis of the SA1 sarcoma.

It can be seen in Fig. 3 that 2 mg of cortisone acetate given intradermally to mice bearing a day 9 SA1 sarcoma had no effect on the ability of 10^5 U of rTNF given intravenously 12 h later to cause hemorrhagic necrosis and subsequent complete tumor regression.

Glucocorticoid Treatment Given after the Hemorrhagic Reaction Does Not Prevent Endotoxin from Causing Tumor Regression. It was shown above that the ability of rTNF to cause hemorrhagic necrosis and subsequent regression of the SA1 sarcoma was not affected by pretreating the host with glucocorticoids. However, it is known (4, 13) that complete regression of this tumor requires that the host possesses an adequate level of concomitant antitumor immunity. This means that glucocorticoid treatment does not inhibit host antitumor immunity. It was anticipated, therefore, that glucocorti-

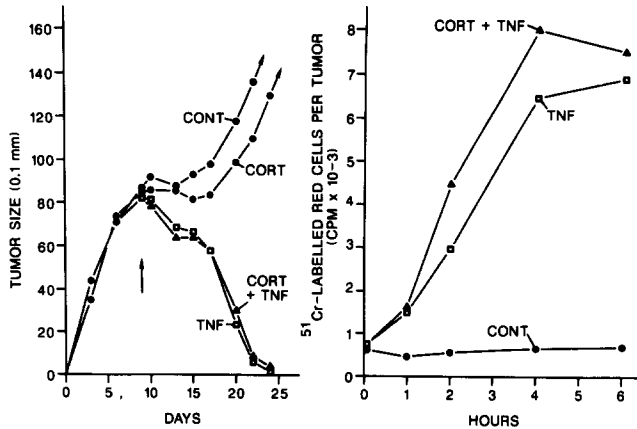


FIGURE 3. Evidence that the ability of 10^5 U of rTNF (TNF) given intravenously on day 9 of tumor growth to cause intratumor hemorrhaging (*right panel*) and tumor regression (*left panel*) was not inhibited by cortisone acetate (CORT + TNF) given 12 h earlier. Means of five mice per group.

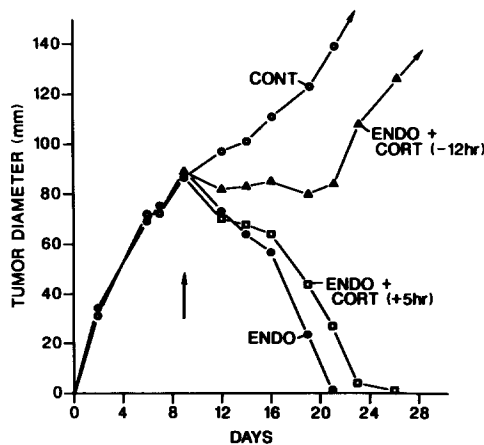


FIGURE 4. Cortisone acetate given intradermally before (ENDO + CORT, -12 h), but not after (CORT + ENDO, + 5 h) endotoxin on day 9 of tumor growth, inhibited endotoxin-induced tumor regression (ENDO). Means of five mice per group.

teroid treatment would not prevent endotoxin-induced tumor regression if given after endotoxin has already caused a hemorrhagic reaction in the tumor.

It was found (Fig. 4) that, whereas cortisone acetate given 12 h before endotoxin inhibited tumor regression, cortisone acetate given 5 h after endotoxin, when the tumor hemorrhagic reaction was almost complete, was without effect. It is apparent, therefore, that cortisone acetate inhibits endotoxin-induced regression solely by inhibiting hemorrhagic necrosis.

Discussion

This study shows that the ability of endotoxin to cause hemorrhagic necrosis and subsequent regression of an established subcutaneous SA1 sarcoma is inhibited by treating the tumor-bearing mice with glucocorticoids 12 h before giving them endotoxin. Inhibition of endotoxin-induced hemorrhagic necrosis was shown to be associated with inhibition of endotoxin-induced TNF production in the tumor and in the host at large. On the other hand, glucocorticoid treatment had no effect on the ability of exogenous TNF to cause hemorrhagic necrosis and regression of the SA1

sarcoma. Given the published evidence showing that TNF is the primary mediator of endotoxin-induced tumor hemorrhagic necrosis (4), the results presented here strongly suggest that glucocorticoids inhibit the antitumor action of endotoxin by inhibiting its ability to induce the production of TNF.

This is not to say that TNF alone is responsible for endotoxin-induced tumor hemorrhagic necrosis. It may also require the participation of already formed host molecules, such as complement component C5 (15), or of induced molecules, such as IL-1 and other cytokines. However, if other induced molecules are involved, their induction would need to be resistant to the doses of glucocorticoids used here. It is known, in this connection, that glucocorticoids can inhibit the synthesis of IL-1 (16-18), as well as TNF (12), by host cells *in vitro*, both at the level of gene transcription and translation. It seems likely, therefore, that IL-1 is not needed for TNF-mediated tumor hemorrhagic necrosis. Even so, a role for IL-1 and other induced cytokines cannot be excluded until it is formally demonstrated that they are not involved.

As for TNF itself, it is likely that intratumor macrophages are mainly responsible for its production in the tumor. It is known that macrophages are major producers of TNF (8), that these cells can be well represented in tumors (9), and that TNF appears to be responsible, at least in part, for the ability of macrophages to kill certain tumor cells *in vitro* (10, 11). It seems almost certain, however, that TNF does not cause tumor hemorrhagic necrosis by directly destroying tumor cells *in vivo*. On the contrary, it was elegantly demonstrated many years ago (18) that endotoxin-induced tumor hemorrhagic necrosis is caused by a breakdown in the tumor's vasculature and consequently in ischemic death of most of the central part of the tumor. This could result from a direct cytotoxic action of TNF on vascular endothelial cells, as has shown to be the case in other models of TNF-induced hemorrhagic necrosis (19). In the study presented here intratumor hemorrhaging was measured in terms of the intratumor extravasation of ^{51}Cr -labeled syngeneic red blood cells infused intravenously shortly before endotoxin or TNF was given.

It needs to be pointed out that the endotoxin-induced vascular breakdown and ischemic death of the center of the tumor is not sufficient to cause complete tumor regression. In order for the rim of living tumor tissue that survives hemorrhagic necrosis to undergo complete regression, it is necessary for the host to be in the process of generating an adequate level of concomitant antitumor immunity (4). The finding that glucocorticoids failed to inhibit the ability of exogenous TNF to cause hemorrhagic necrosis, or subsequent tumor regression, means that these compounds did not significantly depress antitumor immunity. Therefore, their ability to inhibit the antitumor action of endotoxin must have been based solely on their ability to inhibit hemorrhagic necrosis. It is apparent, therefore, that hemorrhagic necrosis serves to reduce the tumor burden to a size that can be completely rejected by an otherwise subtherapeutic level of concomitant immunity.

Because the tumor hemorrhagic reaction can be viewed as a tissue destructive inflammatory reaction caused by the local synthesis of TNF, its inhibition by glucocorticoids indicates that it might serve as a useful model to study in more detail the antiinflammatory action of glucocorticoids and antiinflammatory agents in general.

Summary

Intravenous injection of 25 μg of bacterial endotoxin on day 9 of growth of the SA1 sarcoma results in extensive necrosis of the core of this tumor and in its subsequent complete regression. Tumor hemorrhagic necrosis and regression failed to occur in mice that were given a subcutaneous injection of cortisone acetate or dexamethasone 12 h before being given endotoxin. Inhibition of tumor hemorrhagic necrosis and regression by glucocorticoids was associated with inhibition of endotoxin-induced intratumor TNF production that normally takes place several h after endotoxin is given. In contrast, glucocorticoids had no effect on the ability of intravenously injected rTNF to cause tumor hemorrhagic necrosis and regression. The results lend further support to the belief that TNF is the predominant mediator of endotoxin-induced hemorrhagic necrosis of established murine tumors, and that hemorrhagic necrosis is a prerequisite for the immunologically mediated regression that follows.

We thank Ron LaCourse, Lynn Ryan, Lynn Stiles and Debra Duso for expert technical assistance, and Mary Durett for typing the manuscript.

Received for publication 6 March 1989 and in revised form 30 May 1989.

References

1. Shear, M. J. 1944. Chemical treatment of tumors. IX. Reactions of mice with primary subcutaneous tumors to injection of hemorrhagic-producing bacterial polysaccharide. *J. Natl. Cancer Inst.* 4:461.
2. Parr, I., E. Wheeler, and P. Alexander. 1973. Similarities of the antitumor actions of lipid A and double stranded RNA. *Immunology.* 60:367.
3. DiGiacomo, A., and R. J. North. 1987. Subtherapeutic numbers of tumor-sensitized L3T4⁺, Ly1⁺2⁻ T cells are needed for endotoxin to cause regression of an established immunogenic tumour. *Immunology.* 60:367.
4. North, R. J., and E. A. Havell. 1988. The antitumor action of tumor necrosis factor (TNF). II. Analysis of the role of endogenous TNF in endotoxin-induced hemorrhagic necrosis and regression of an established sarcoma. *J. Exp. Med.* 167:1086.
5. Carswell, E. A., L. J. Old, R. L. Kassel, S. Green, N. Fiore, and B. Williamson. 1975. An endotoxin-induced serum factor that causes necrosis of tumors. *Proc. Natl. Acad. Sci. USA.* 72:3666.
6. Shiral, T., H. Yamaguchi, H. Ito, C. W. Todd, and R. B. Wallace. 1985. Cloning and expression of the gene for tumor necrosis factor. *Nature (Lond.).* 313:803.
7. Pennica, D., G. E. Nedwin, J. S. Hayflick, P. H. Seeburg, R. Derynch, M. A. Palladino, W. J. Kohr, B. B. Aggarwal, and D. V. Goeddel. 1984. Human tumor necrosis factor: precursor structure, expression and homology to lymphotoxin. *Nature (Lond.).* 312:724.
8. Old, L. J. 1985. Tumor necrosis factor. *Science (Wash. DC).* 230:630.
9. Evans, R. 1972. Macrophages in syngeneic tumors. *Transplantation (Baltimore).* 14:468.
10. Mannel, D. N., R. N. Moore, and S. E. Mergenhagen. 1980. Macrophages as a source of tumoricidal activity (tumor necrosis factor). *Infect. Immun.* 30:523.
11. Urban, T. L., M. M. Shepard, J. L. Rothstein, B. J. Sugarman, and H. Schreiber. 1986. Tumor necrosis factor: a potent effector molecule for tumor killing by activated macrophages. *Proc. Natl. Acad. Sci. USA.* 83:5233.
12. Beutler, B., N. Krochin, I. W. Milsbark, C. Luedke, and A. Cerami. 1986. Controls of cachectin (tumor necrosis factor) synthesis: mechanisms of endotoxin resistance. *Science (Wash. DC).* 232:977.

13. Havell, E. A., W. Fiers, and R. J. North. 1988. The antitumor function of tumor necrosis factor (TNF). I. Therapeutic action of TNF against an established murine sarcoma is indirect, immunologically dependent, and limited by severe toxicity. *J. Exp. Med.* 167:1067.
14. Wang, A. M., A. A. Creasey, M. B. Ladner, L. S. Lin, J. Strickler, J. N. Van Arsdell, R. Yamamoto, and D. F. Mark. 1985. Molecular cloning of the complementary DNA for human tumor necrosis factor. *Science (Wash. DC)*. 228:149.
15. Rothstein, J. L., T. F. Flint, and H. Schreiber. 1988. Tumor necrosis factor/cachectin. Induction of hemorrhagic necrosis in normal tissue requires the fifth compound of complement (C5). *J. Exp. Med.* 168:2007.
16. Knudsen, P. J., C. A. Dinarello, and T. B. Strom. 1988. Glucocorticoids inhibit transcriptional and postranscriptional expression of interleukin-1 in U937 cells. *J. Immunol.* 139:4129.
17. Lew, W., J. J. Oppenheim, and K. Matsushima. 1988. Analysis of the suppression of IL-1 α and IL-1 β production in human peripheral blood mononuclear adherent cells by glucocorticoid hormone. *J. Immunol.* 140:1895.
18. Lee, S. W., A-P. Tsou, H. Chan, J. Thomas, K. Petrie, E. M. Eugui, and A. C. Allison. 1988. Glucocorticoids selectively inhibit transcription of the interleukin 1 β gene and decrease the stability of interleukin 1 β mRNA. *Proc. Natl. Acad. Sci. USA.* 85:1204.
19. Algire, G. H., F. Y. Legallis, and H. D. Park. 1948. Vascular reactions of normal and malignant tissues *in vivo*. II. The vascular reaction of normal and malignant tissues of mice to bacterial polysaccharide from *Serratia marcescens* (*Bacillus prodigeosus*) culture filtrates. *J. Natl. Cancer Inst.* 8:53.
20. Grau, G. E., P-F. Piguet, P. Vassalli, and P.-H. Lambert. 1989. Involvement of tumor necrosis factor and other cytokines in immune-mediated vascular pathology. *Int. Arch. Allergy Appl. Immunol.* 88:34.