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SEPARATION OF A SERUM-DERIVED TUMORICIDAL FACTOR FROM A HELPER FACTOR FOR PLAQUE-FORMING CELLS

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C3H/HeN mice administered BCG followed by lipopolysaccharide 14 days later released into their serum a cytotoxic factor for tumor cells and a factor that restored the anti-SRBC plaque-forming cell response of nude mouse spleen cells (helper activity). Gel filtration of serum containing the cytotoxic and the helper activities indicated that both factors exhibited an apparent m.w. of 125,000 to 150,000. The helper activity was also found at lower m.w. (60,000 and 13,000) suggesting the possibility that this factor existed in aggregated forms. Gel filtration of ammonium sulfate (40 to 60% saturation) precipitated serum in a high ionic strength buffer (1.6 M NaCl) resulted in shifts in the apparent m.w. of both factors. The cytotoxic factor now exhibited a m.w. of 55,000. The helper activity eluted with an apparent m.w. of 13,000, and thus was clearly separated from the cytotoxic factor. The helper activity was further shown to co-elute with macrophage-derived lymphocyte activating factor (LAF). This as well as other data represent the first demonstration of *in vivo* produced LAF.

Administration of lipopolysaccharide (LPS) from Gram-negative bacteria to bacillus Calmette-Guérin-(BCG)[2] infected mice induces the release into the serum of a number of biologically active soluble products, including colony-stimulating factor (1), interferon (2), tumor necrosis factor (TNF) (3–6), and PFC helper factor (3). In addition, the serum exhibits marked cytotoxic effects on tumor cells grown in culture (4, 7, 8). This cytotoxic effect has generally been considered to represent an *in vitro* assay for TNF (7, 8). Recently, Hoffmann *et al.* (3, 6) reported that the PFC helper factor and TNF co-purified after ammonium sulfate precipitation, gel filtration, and polyacrylamide gel electrophoresis, suggesting that both the helper and cytotoxic activities might be mediated by the same molecular species. In the course of our investigations on the isolation and purification of LPS-induced cytotoxic factor (CF) for tumor cells, we also observed that serum from BCG-primed and LPS-treated mice contained not only tumoricidal activity but also PFC helper activity. In this report, we demonstrate that the helper factor and the CF are distinct molecular entities. We further show that the helper factor co-purifies with a soluble factor that enhances the proliferative response of thymocytes to PHA and is probably identical with macrophage-derived lymphocyte activation factor (LAF; Interleukin 1 (IL 1)).[3]

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[2] Abbreviation used in this paper: BCG, bacillus Calmette-Guérin; CF, cytotoxic factor; DMEM, Dulbecco's modified Eagle's minimal essential medium; EMEM, Eagle's minimal essential medium; LAF, lymphocyte-activating factor; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

MATERIALS AND METHODS

Mice. C3H/HeJ mice were obtained from The Jackson Laboratory, Bar Harbor, Maine, C3H/HeN mice and C57BL/10ScN nude mice were provided by the Division of Research Resources, National Institutes of Health, Bethesda, Md., and BALB/C nude mice were purchased from ARS Sprague-Dawley, Madison, Wis. Male and female mice 6 to 15 weeks of age were used in all experiments.

Bacillus Calmette-Guérin. Mycobacterium bovis, strain BCG (Phipps substrain TMC No. 1029), was obtained from the Trudeau Mycobacterial Collection, Saranac Lake, N. Y.

Lipopolysaccharide. Escherichia coli, K-235 LPS was prepared by the phenol-water extraction method of McIntire *et al.* (9).

Generation of BCG-LPS serum. C3H/HeN mice were injected i.v. with 2×10^6 colony-forming units of living BCG. Two weeks later the mice were injected i.v. with 10 μg of LPS. The mice were exsanguinated 2 hr later, the serum was sterilized by filtration and stored at −20°C until further use (BCG-LPS serum). Control sera from mice injected with BCG (BCG serum) or LPS without prior BCG infection (LPS serum) were similarly prepared.

Cytotoxic factor assay. Tumor cells (mouse L cells strain 929) were seeded in 16-mm culture wells (Costar No. 3524) (4×10^4 cells/well) and labeled in 0.5 ml Eagle's minimal essential medium (EMEM) with 25 mM HEPES (see Abbreviations) buffer, 5% heat-inactivated (30 min, 56°C) fetal calf serum (FCS), and 0.5 μl/ml [^{3}H]TdR (methyl-(^{3}H)-thymidine, sp. act. 1.9 Ci/mmol, Schwarz/Mann, Orangeburg, N. Y.) for 18 to 24 hr. Tumor cell monolayers were washed twice after labeling and incubated with 1 ml of Dulbecco's modified Eagle's minimal essential medium (DMEM) HG (4.5 g glucose/liter; 2 g $NaHCO_3$/liter, 10% heat-inactivated FCS, and 50 μg gentamycin/ml) with different dilutions of BCG-LPS and control serum. Labeled tumor cell monolayers lysed with 0.5% sodium dodecyl sulfate in water were used to estimate total incorporated counts. Cytotoxicity was determined by measuring release of incorporated [^{3}H]TdR from prelabeled tumor cells in duplicate cultures.

[3] A revised nomenclature for lymphocyte-activating factor (LAF) was proposed at the recent Second International Lymphokine Workshop (Ermatingen, Switzerland, May 27–31, 1979). The revised name for LAF is Interleukin 1. To avoid confusion, the term IL 1 will be initially assimilated into the literature by using both acronyms as follows: LAF (IL 1). With time, the term LAF will be deleted.

PFC helper factor assay. Dilutions of BCG-LPS and control sera were assayed for their ability to restore the *in vitro* anti-SRBC PFC response of nude mouse spleen cells as previously described (10), with the exception that the cells were cultured in the presence of 1% normal mouse serum rather than FCS.

Thymocyte proliferation assay. Dilutions of BCG-LPS serum and control serum were assayed for their ability to enhance the proliferative response of C3H/HeJ mouse thymocytes to 1.0 μg/ml PHA as described previously (10).

Ammonium sulfate precipitation of serum. Ten milliliters of both BCG-LPS and control sera were precipitated by $(NH_4)_2SO_4$. Sufficient $(NH_4)_2SO_4$ was slowly added to the stirring serum samples at 4°C to achieve 40% $(NH_4)_2SO_4$ saturation. The precipitate was then stirred for 1 hr, followed by centrifugation at 10,000 × G for 15 min. The precipitate was dissolved in pH 7.5 (4°C) Tris-buffered saline (0.05 M Tris, 0.1 M NaCl). The precipitation, centrifugation, and redissolving of the precipitate were then repeated with the supernatant to prepare 40 to 60%, 60 to 80%, and 80 to 100% $(NH_4)_2SO_4$ saturation precipitated samples. All of the samples were dialyzed extensively against Tris-buffered saline.

Gel filtration. Up to 10 ml of either unfractionated or ammonium sulfate-precipitated serum samples were subjected to gel filtration on a 2.5 x 90 cm calibrated Sephacryl S-200 column that had been equilibrated in pH 7.5 (4°C) Tris (0.05 M) buffer containing either 0.1 M or 1.6 M NaCl. All chromatography was performed at 4°C by using reverse flow at a flow rate of approximately 20 ml/hr. Fractions of 2.5 to 2.8 ml were collected. Each fraction was individually and extensively dialyzed against tissue culture medium. Normal mouse serum was added to each fraction to a concentration of 1%, and the fractions were sterilized by filtration and assayed for CF, PFC helper activity, and thymocyte mitogenic activity as described above.

Protein determination. Protein content of BCG-LPS serum, LPS serum, and partially purified fractions were determined by using the Bio-Rad protein assay kit (Bio-Rad, Richmond, Calif.).

RESULTS

Serum from BCG-infected mice challenged with LPS (BCG-LPS serum) and control sera (LPS serum and BCG serum) were tested for their cytotoxic and PFC helper activity. The results of a representative experiment are shown in Figure 1. The cytotoxic activity of BCG-LPS serum for murine tumor cell targets was observed at dilutions ranging from 10^{-2} to 5×10^{-4}. High tumoricidal activity was consistently found at a dilution of 1:100. The BCG-LPS serum exhibited a similar dose-dependent enhancing effect on the PFC response. Significant helper activity was detected at dilutions as low as 1:1280. The controls consisting of serum from either LPS- (shown) or BCG- (not shown) treated mice failed to exhibit either cytotoxic or PFC helper activity.

In order to separate the cytotoxic activity from the PFC-promoting activity (helper factor), BCG-LPS and LPS sera were subjected to gel filtration on a Sephacryl S-200 column, and the fractions were assayed for CF and helper factor (Fig. 2). CF consistently eluted as a single peak with maximal activity at a m.w. of approximately 150,000. In this experiment, the helper activity eluted as one major peak with an apparent m.w. of 60,000 and two minor peaks with apparent m.w. of approximately 125,000 and 13,000. In other experiments, the principal helper activity peak was observed to co-elute with CF in the m.w. range of 125,000 to 150,000. Thus, although the PFC helper factor may elute in the m.w. range of CF (125,000 to 150,000), these data suggest that the helper factor exists in aggregated forms, and that separation of the helper factor from CF by gel filtration is dependent on the deaggregation of the helper factor.

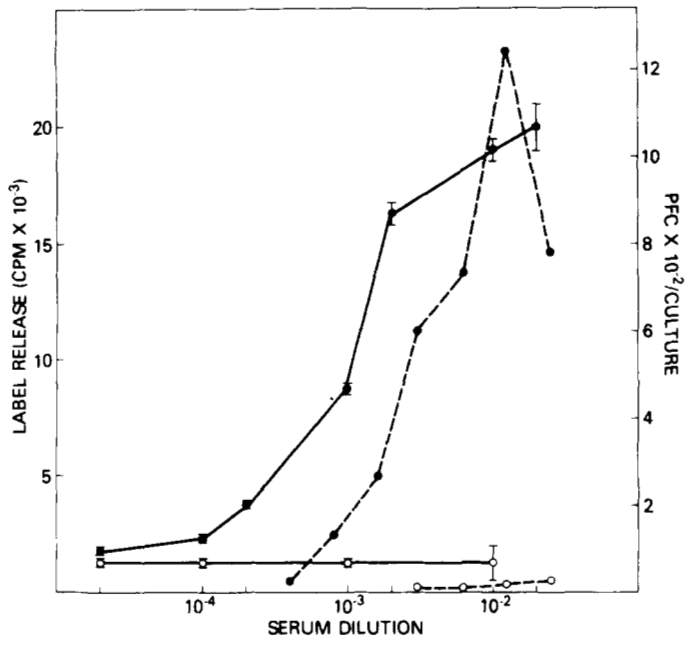

Figure 1. Dose responses of BCG-LPS serum and control serum for tumor cytotoxicity and PFC helper activity. Tumor cytotoxicity was estimated by [^{3}H] TdR release of labeled L 929 cells after 48 hr of incubation in different dilutions of BCG-LPS serum (*closed circles*) or control serum (*open circles*) (total incorporated cpm = 45×10^3) (*solid lines*). Plaque-forming cell helper activity was determined in the anti-SRBC plaque assay with nude mouse spleen cells by adding different dilutions of BCG-LPS serum (*closed circles*) or control serum (*open circles*) to the cultures at day 0 (*dashed lines*).

In order to concentrate larger volumes of serum in preparation for gel filtration, the proteins in the BCG-LPS and control sera were precipitated with ammonium sulfate. The CF and helper activities present in the sequential ammonium sulfate precipitates are shown in Figure 3. The results show that approximately 50% of the precipitated protein and virtually 100% of the CF and helper activities are present in the 40 to 60% ammonium sulfate saturation precipitate. The precipitates of the control serum from LPS-treated mice exhibited neither CF nor helper activity. Subsequent ammonium sulfate precipitation experiments demonstrated that most of the CF and helper activities were precipitated between 45 to 50% ammonium sulfate saturation (data not shown). When this latter precipitated fraction was subjected to gel filtration under conditions of high ionic strength, the elution profiles of CF and helper factor shown in Figure 4 were obtained. Panel A shows that under these chromatographic conditions, CF elutes at an apparent m.w. of 55,000, whereas the helper factor (Panel B) now eluted at a m.w. of 13,000. No helper activity was found in higher m.w. fraction pools. Thus, under these conditions, both CF and the helper factor were found to elute as single peaks with distinct m.w. Because of the similarity in the m.w. of the helper factor and that reported for LAF (IL 1) (11–13), which also has been shown to enhance the antibody response of T cell-deficient mouse spleen cells (14–16), the lower m.w. fractions containing the helper activity were also assayed for their ability to enhance thymocyte proliferation in a standard LAF

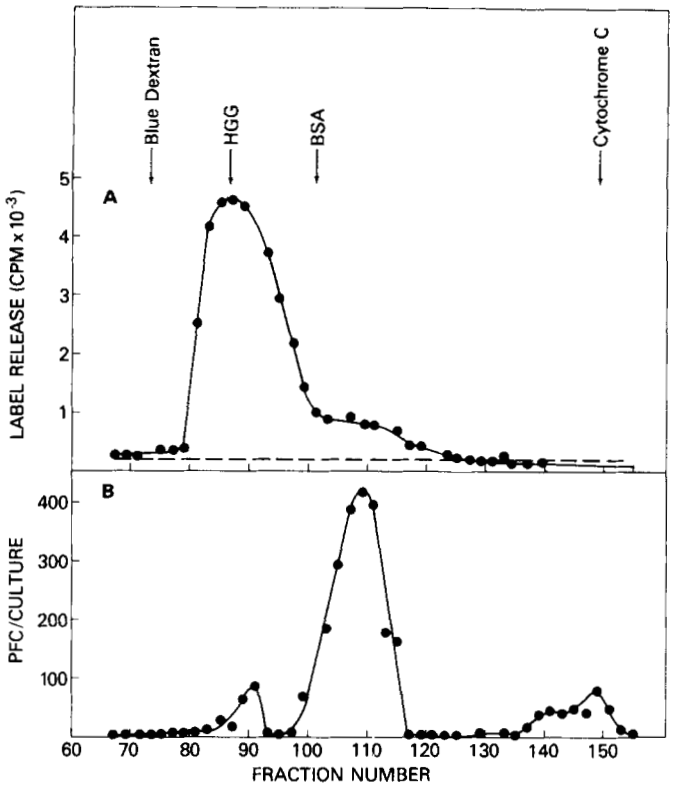

Figure 2. Sephacryl S-200 chromatography of BCG-LPS serum and control serum. Two milliliters of unfractionated BCG-LPS serum (*solid line*) or control serum (*dashed line*) were subjected to Sephacryl S-200 gel filtration. The column was equilibrated and eluted with Tris buffer (pH 7.5) containing 0.1 M sodium chloride. Fractions of 2.5 ml were collected. Tumor cytotoxic activity (*Panel A*) and PFC helper activity (*Panel B*) were estimated as described in *Materials and Methods.*

(IL 1) assay (Panel C). A comparison of Panels B and C demonstrate that the fractions containing helper activity are also mitogenic for mouse thymocytes, maximal activity in both assays appearing in the identical fraction. As seen previously, none of the fractions from the control LPS serum exhibited CF, PFC helper factor, or thymocyte mitogenic activity.

In addition to the negative results obtained with the control serum (LPS-induced serum), we tested the possibility that the PFC helper activity in BCG-LPS serum fractions was due to a direct LPS effect. Accordingly, we compared the helper activity of a pool of low molecular BCG-LPS serum fractions containing LAF (IL 1) and LPS itself in cultures containing LPS-responsive and nonresponsive nude mouse spleen cells (Fig. 5). The data in Panel A demonstrates that the PFC response of both LPS responsive (BALB/C) and nonresponsive (BL/10ScN) nude mouse spleen cells is restored by the addition of partially purified serum-derived helper factor from BCG- and LPS-treated mice. In contrast, the data in Panel B shows that the PFC response of the LPS-responsive nude mouse spleen cells, but not the nonresponsive nude mouse spleen cells, is enhanced by the addition of LPS to the cultures.

DISCUSSION

Recently, Hoffmann *et al.* (3) reported that serum from BCG-infected mice challenged with LPS contained tumor necrosis factor and a PFC helper factor, and that a partially purified preparation contained both activities. The m.w. described for both activities was reported to be approximately 150,000. We have similarly found that cytotoxic factor and helper activity to exhibit similar apparent m.w. (125,000 to 150,000) when unfractionated serum is chromatographed under conditions that do not favor molecular deaggregation (data not shown). However, when the BCG-LPS serum was first precipitated with ammonium sulfate and then subjected to gel filtration in high ionic strength buffer, the cytotoxic serum factor was clearly separable from the helper activity (Fig. 4). Thus, the helper factor in LPS-induced serum from BCG-treated mice appears to be a distinct molecular species from the CF. The apparent m.w. of the CF (55,000) obtained in gel filtration experiments with the high ionic strength buffer is in close agreement with the m.w. estimate (50,000) for a rabbit cytotoxic factor (designated TNF by the authors) (8). Recent observations from our laboratory show that the activated macrophage is the cellular source for CF (D. Männel and S. Mergenhagen, manuscript in preparation) as it was proposed for TNF (4). Our observation that the PFC helper activity in BCG-LPS serum exhibits a m.w. of 13,000 is of interest, in view of the fact that the principal macrophage-derived helper factor produced *in vitro* after stimulation with a variety of agents, including LPS, also exhibits a similar m.w. (15, 16). Moreover, similar to what has been observed with *in vitro*-produced macrophage-derived helper factor (15, 16), the factor present in LPS-induced serum from BCG-infected mice also exhibits the capacity to enhance the thymocyte proliferative response (Fig. 4). Thus, by a number of criteria, including: 1) the m.w., 2) biologic activities, 3) inductive stimulant, and 4) presumptive cell of origin (the activated macrophage (13, 14, 16), the PFC helper factor present in BCG-LPS serum is presumably identical to LAF (IL 1). This represents the first demonstration of *in vivo*-produced LAF (IL 1) activity.

Numerous reports have shown that activated macrophages as well as tumor-specific cytotoxic T cells provide important host defense mechanisms against tumors (17). Furthermore, recent experiments indicate that LPS may serve to enhance

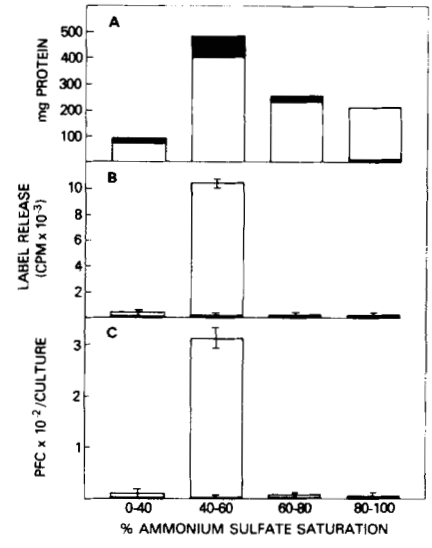

Figure 3. Ammonium sulfate precipitation of BCG-LPS serum and control serum. Ten milliliters of BCG-LPS serum (*open bars*) or control serum (*solid bars*) were precipitated with ammonium sulfate in increasing increments of concentration. For each fraction, the amount of precipitated protein was determined (*Panel A*), the tumor cytotoxic activity (in 1:1000 dilution, total incorporated cpm = 30×10^3) (*Panel B*) and PFC helper activity (in 1:320 dilution) (*Panel C*) were estimated as described in *Materials and Methods.*

both the nonspecific and specific anti-tumor host defense mechanisms (18, 19). For example, Russel *et al.* (18) have shown that primed macrophages from tumors can be triggered to become cytotoxic with very low concentrations of LPS *in vitro*. Similarly, Berendt *et al.* (19, 20) have shown that administration of LPS to tumor-bearing mice initiates antigen-specific T cell-dependent regression of the tumors. Because the activation of antigen-specific cytotoxic T cells in this latter system as well as in numerous other *in vitro* systems has been shown to be macrophage dependent (19, 20), the LPS-activated macrophage is presumably crucial to the antigen-specific as well as the nonspecific mechanisms of tumor immunity. Thus, LPS apparently exhibits a bimodal anti-tumor effect; being involved in macrophage-activation processes that culminate in enhanced specific and nonspecific host defense mechanisms.

In this regard, it is of interest that we were able to identify

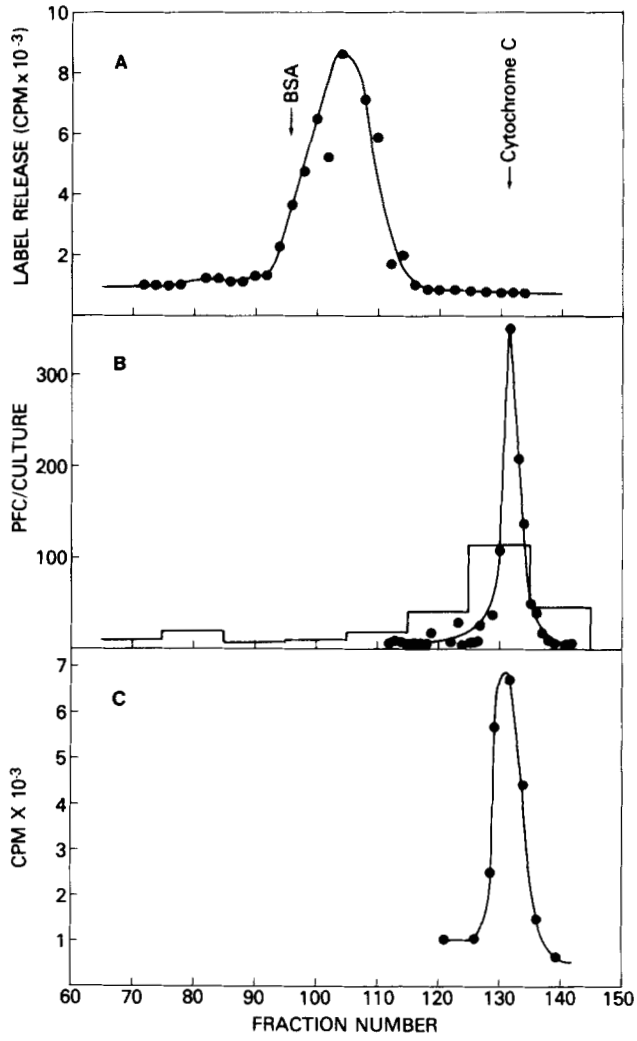

Figure 4. Sephacryl S-200 chromatography of ammonium sulfate precipitated BCG-LPS serum. The 45 to 50% ammonium sulfate precipitate from 10 ml of BCG-LPS serum was subjected to Sephacryl S-200 gel filtration on a 2.5- x 90-cm column. The column was equilibrated and eluted with Tris buffer (pH 7.5) containing 1.6 M sodium chloride. Fractions of 2.7 ml were collected. Tumor cytotoxic activity (*Panel A*), PFC helper activity (*Panel B*), and thymocyte proliferative activity (*Panel C*) were assayed as described in *Materials and Methods*. For the PFC helper activity, pools of 10 fractions each were assayed first and then individual fractions were tested in the region where the activity was located.

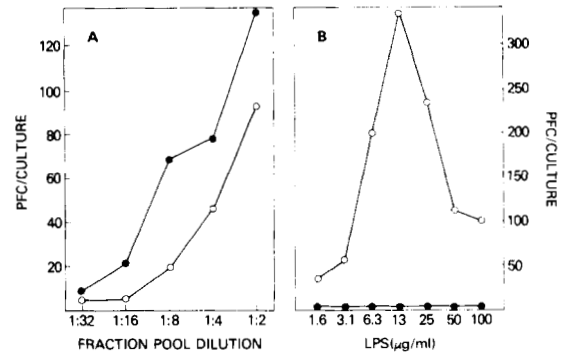

Figure 5. Comparison of LPS responsive and nonresponsive nude mouse spleen cells to the PFC helper activity of BCG-LPS serum derived LAF (IL 1) and LPS. The fractions which had PFC helper activity and LAF (IL 1) activity after ammonium sulfate precipitation and Sephacryl S-200 chromatography were pooled and assayed in different dilutions for their ability to restore the anti-SRC plaque-forming response of LPS responsive (BALB/C) nude mouse spleen cells (*open circles*) and LPS nonresponsive (BL/10ScN) nude mouse spleen cells (*closed circles*) (*Panel A*). In parallel, the ability of *E. coli* K 235 LPS to restore the plaque-forming response was determined in the same assay with both mouse strains (*Panel B*).

and separate two putative macrophage-derived soluble products in the LPS-induced serum from BCG-infected mice that might provide the mediators for the nonspecific and specific LPS effects. CF would presumably be involved in a nonspecific tumoricidal (tumor necrosis) effect. This nonspecific effect is apparently T cell independent, in that CF is produced in nude mice and the tumor necrosis effect of LPS can be observed in tumor-bearing T cell-deficient mice (19). In contrast, we would suggest that LAF (IL 1), which we were detecting as an enhancer of the PFC response, might also be involved in the induction of a T cell-dependent antigen-specific anti-tumor response. This hypothesis is supported by the observation that highly purified LAF (IL 1) is capable of replacing the requirement for macrophages during the *in vitro* induction of alloantigen specific cytotoxic T cells (21). These data are totally consistent with the observations of Berendt *et al.* (19), who have shown that LPS-induced tumor regression is dependent upon: 1) the immunogenicity of the tumor, and 2) collaborative interactions between macrophages and T cells.

Further characterization of the immunologically active factors in BCG-LPS serum and their application to *in vivo* experiments should help to clarify the relationship between the specific and nonspecific anti-tumor effects of LPS-activated macrophages.

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