The Immunosuppressive Effect of Dactinomycin on Experimentally Induced Granulomas—A Quantitative Study[1] (37290)

STEVEN D. PAUL,[2] THOMAS J. ATHANASSIADES,[3] AND ROBERT S. SPEIRS[4]
Departments of Anatomy and Pathology Downstate Medical Center, Brooklyn, New York 11203

Our laboratory has been concerned with the experimentally induced granuloma as a model for the study of cell responses to antigenic materials and the relationship of these responses to the immunological status of the host (1, 2). These studies have indicated that the cellular content of a granuloma, induced with alum-localized antigen, reflected the ability of an organism to respond to an immunological stimulus. Furthermore, it was demonstrated that irradiated syngeneic hosts receiving a granuloma transplant acquired the capacity to synthesize antibody as well as the ability to mount an anamnestic response when challenged with specific antigen (1). These studies suggested that the local granuloma possessed immunological memory and the capacity for antibody synthesis. Therefore, it was considered to represent a local manifestation of the immune response, and to reflect the capacity of the lymphoid and reticuloendothelial systems of the host to respond to antigenic stimulation.

In order to achieve a more accurate estimate of the number and types of cells comprising the granuloma, a procedure was devised for measuring the granuloma by means of a quantitative cell analysis (3). This method made it possible to categorize granulomas into a variety of types depending upon the nature of the antigenic material utilized, and the immunological sensitivity of the host to the inciting antigen. It was decided that this method might be used for a quantitative study of the effect of immunosuppressive agents on the cellular content of developing granulomas.

The investigation to be reported here involves the study of the effect of Dactinomycin on the cellular content of antigen-induced granulomas, and the relationship of its effect to the production of circulating antibody in primed and unprimed animals.

Materials and Methods. Animals. The experimental animal used throughout this study was the adult female BDF_1 mouse (Jackson Memorial Laboratories, Bar Harbor, Maine), the F_1 of a cross between DBA/2 males and C57 Bl/6 females.

Mice used for assaying the titers of tetanus antitoxin were Swiss albinos, supplied by the New York State Department of Health, Albany.

Dactinomycin. Dactinomycin (Dact) (Merck Sharpe and Dohme) received as a lyophilized powder was reconstituted with pyrogen-free water and diluted to the desired concentration with Hanks' balanced salt solution (HBSS).

Mice received a single intraperitoneal (ip) injection of either 0.60 mg/kg of Dact (4), or HBSS as control, 24 hr after granuloma induction in the inguinal region.

Granuloma induction. Mice were divided into three major groups, each treated according to a specific injection scheme (Table I). All mice received two separate subcutaneous injections given 5 weeks apart. The first was given in the dorsal neck region (N), and the

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[2] Submitted in partial fulfillment for the degree of Doctor of Philosophy, Department of Anatomy, Downstate Medical Center, Brooklyn, New York 11203.

[3] Thomas J. Athanassiades, Associate Professor of Pathology, Downstate Medical Center, Brooklyn, New York 11203.

[4] Robert S. Speirs, Professor of Anatomy and Career Scientist of the Health Research Council of the City of New York at the Department of Anatomy, Downstate Medical Center, Brooklyn, New York 11203.

TABLE I. Experimental Design.

Group	Subcutaneous injection[a]		Designation of inguinal granuloma[b]
	Dorsal neck	Inguinal	
I	PV-TT	AP	Alum—Dact[c] Alum—HBSS
II	PV-AP	TT	TT-primary—Dact TT-primary—HBSS
III	PV-TT	TT	TT-secondary—Dact TT-secondary—HBSS

[a] The dorsal neck injection was given 5 weeks prior to the inguinal injection.

[b] Studies were performed only on the inguinal granulomas.

[c] 0.60 mg/kg of Dactinomycin (Dact) in Hanks' balanced salt solution (HBSS) was injected intraperitoneally 24 hr following the inguinal injection.

second was given in the inguinal region (I). Group I received as their first injection a suspension of 0.2 ml of aluminum phosphate adsorbed tetanus toxoid (APTT) (Parke Davis) mixed with an adjuvant of 0.2 ml of pertussis vaccine (PV) (Eli Lilly) diluted 1:10 with saline. The second injection consisted of 0.4 ml of aluminum phosphate (AP) (5 mg/ml of saline). Group II received an initial injection of 0.2 ml of AP mixed with 0.2 ml of PV adjuvant. Five weeks later, they received an inquinal injection of 0.4 ml of APTT. Group III received as their first injection a suspension of 0.2 ml of APTT mixed with 0.2 ml of PV adjuvant followed five weeks later with a challenging injection of 0.4 ml of APTT.

In order to facilitate location of granulomas, activated sterile carbon (C) (Merck and Co.), in a concentration of 2 mg/ml, was mixed with all suspensions just prior to injection.

Autopsy procedures and processing of granulomas. Five to nine mice for each group were sacrificed on Days 2, 4, 7, 14, and 28 after inguinal injection. Granulomas were removed, trimmed of fat, and subjected to dispersion and digestion procedures for quantitative cell analysis.

The quantitative procedure for the study of the cells in experimentally induced granulomas has been described (3). Briefly, granulomas were subjected to dispersion and digestion in 1.1 ml of a solution of collagenase (4 mg) (General Biochemical Corp.) and pronase (1 mg) (Grade B, Calbiochem, Los Angeles, CA), in HBSS (pH 7.1–7.2). Total cell and eosinophil counts were performed on the resulting cell suspension using Speirs–Levy eosinophil counting chambers (C. A. Hausser, Philadelphia, PA). A sample of the cells was spread onto slides, fixed in methanol, and stained with May–Gruenwald Giemsa blood stain. Differential cell counts were made and the total number of each cell type estimated.

Cells were classified as neutrophils, eosinophils, mononuclear cells (fibrocytes, macrophages, and lymphocytes), basophilic mononuclear cells (dark blue staining mononuclear cells), and plasma cells. Mast cells and giant cells were consistently present in very small numbers and were not graphically represented.

Procedures for preparing granulomas for electron microscopy have been described previously (1).

Antitoxin determination. A quantitative bioassay procedure, modified after Ipsen (5), was used to determine the antitoxin titers. An 0.1 ml of tail vein blood, obtained 1 day before and 28 days after inguinal injection was hemolyzed with 1.9 ml of sterile pyrogen-free water and serially diluted with stock toxin solution. The antitoxin titer of the diluted samples was determined by noting the greatest dilution which protected against the lethal effects of the toxin.

Results. I. Quantitative cell analysis. A. Total cell response (Fig. 1). The effect of

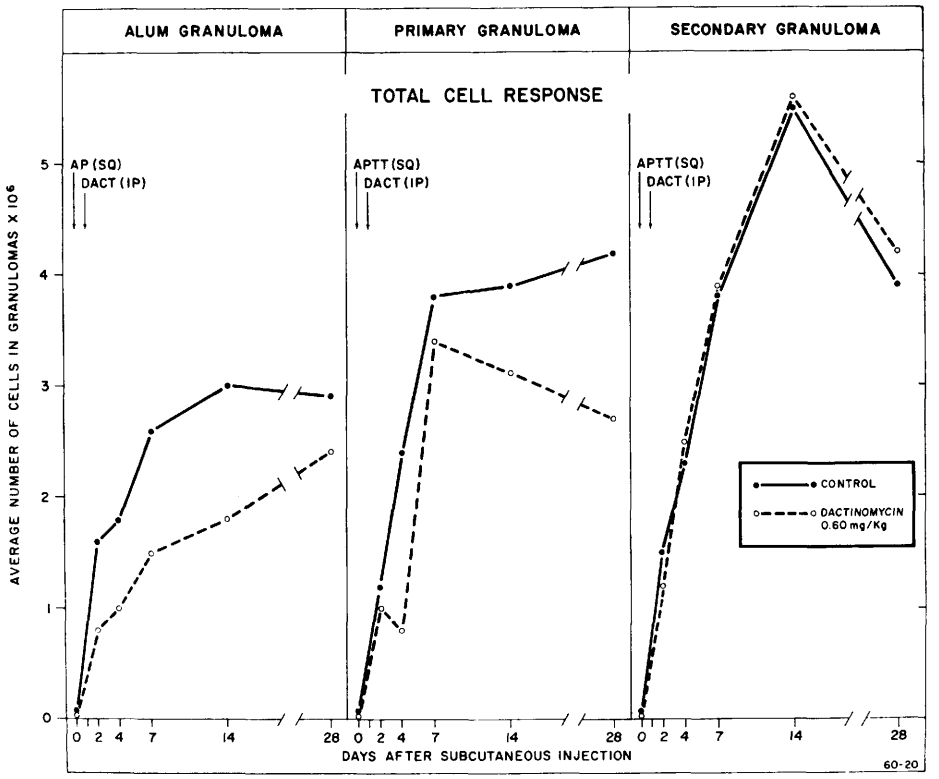

FIG. 1. Effect of Dactinomycin on the total number of cells present in alum, TT-primary, and TT-secondary granulomas induced subcutaneously in the inguinal region. Each point represents 5–9 mice. (TT, tetanus toxoid).

Dact on the total number of cells present in developing alum and TT-primary granulomas was evident as early as 24 hr after treatment. The total number of cells in both alum and TT-primary granulomas was consistently lower than control values throughout the experimental period. In contrast, Dact had no effect on the total number of cells present in TT-secondary granulomas, since identical patterns of response occurred in both drug-treated and untreated groups.

B. Differential cell response (Fig. 2). 1. Neutrophil response. The number of neutrophils in alum granulomas from Dact-treated mice was lower than that present in animals treated with HBSS on all autopsy days. On Day 4, in the TT-primary granulomas, the average number of neutrophils was significantly lower ($p < 0.02$) in Dact-treated mice. The number of neutrophils in TT-secondary granulomas from Dact-treated mice was essentially similar to control values on all days tested.

2. Eosinophil response. The average number of eosinophils present in all three granuloma types from Dact-treated mice was not significantly different ($p > 0.02$) from controls at Days 2, 4, 7, and 14. Increased variation between groups was observed on Day 28.

3. Mononuclear cell response. Alum granulomas from Dact-treated mice showed a consistently lower mononuclear cell response. This was also true for TT-primary granulomas, with the greatest difference occurring on Day 4. The mononuclear cell population in the TT-secondary granulomas was not inhibited by Dact treatment.

4. Basophilic mononuclear cell response. Mononuclear cells with a marked degree of cytoplasmic basophilia have been described previously in granulomatous tissue (3) as

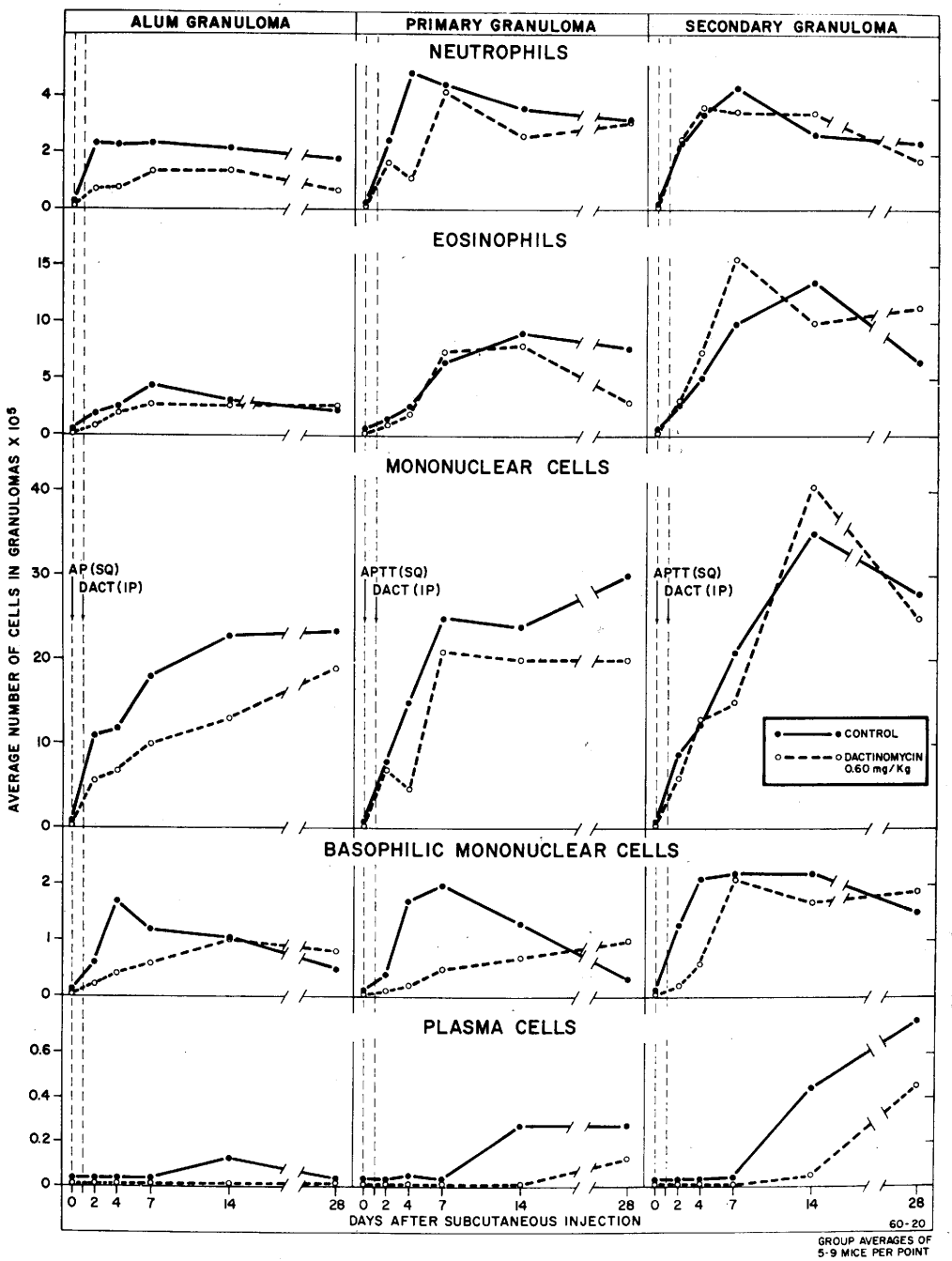

FIG. 2. Effect of Dactinomycin on the total number of each cell type present in alum, TT-primary, and TT-secondary granulomas induced subcutaneously in the inguinal region. Each point represents 5–9 mice. (TT, tetanus toxoid).

well as in lymphatic tissue undergoing antigentic stimulation (6) (Fig. 3).

In Dact-treated mice, a significant decrease ($p < 0.01$) in the basophilic mononuclear cell response occurred on Days 2 and 4 in all groups. After Day 4, the effect of Dact

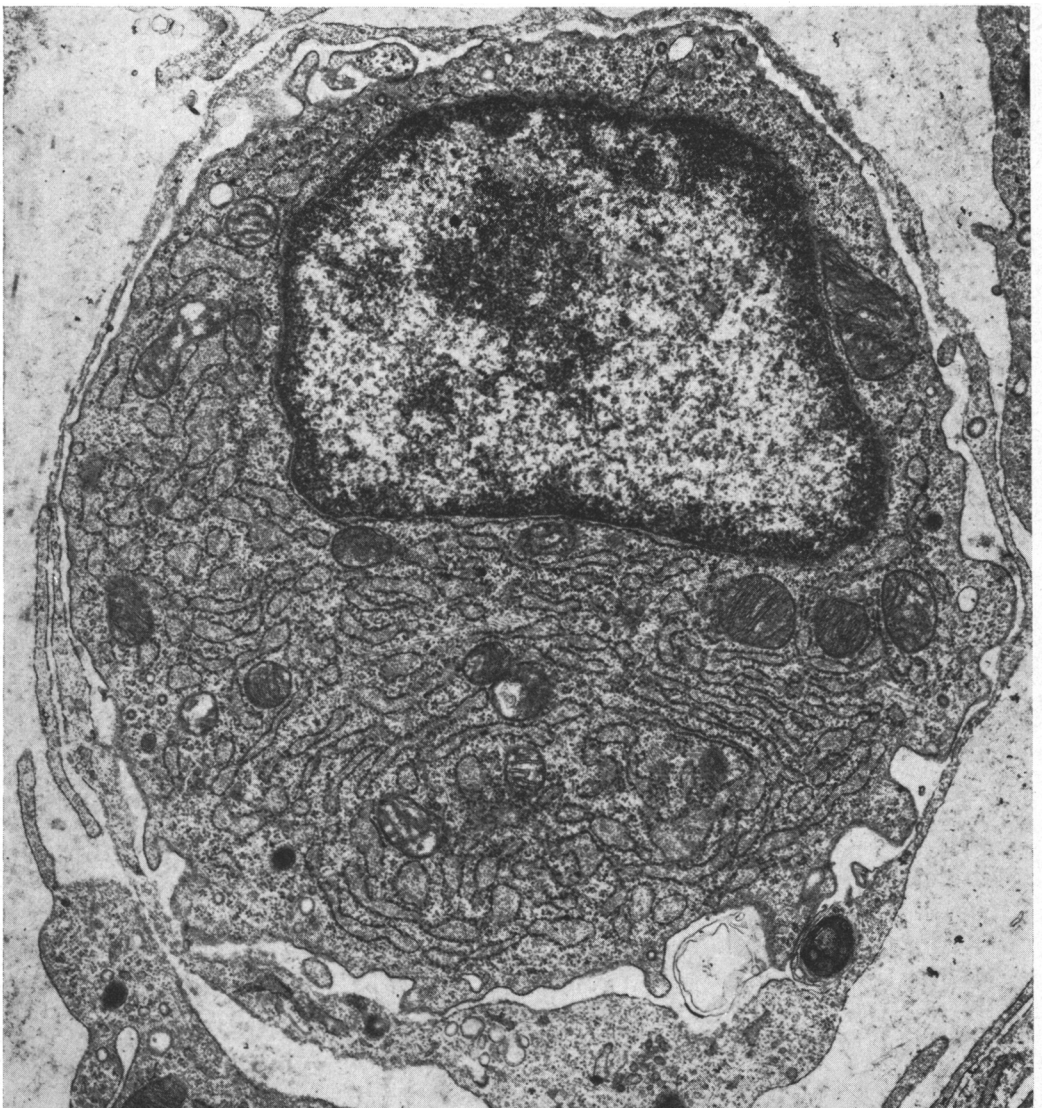

FIG. 3. Electron micrograph of the ultrastructural counterpart of a basophilic mononuclear cell with rough endoplasmic reticulum and free ribosomes. (×42,000).

varied depending upon the granuloma type. The number of basophilic mononuclear cells in the Dact-treated mice was identical in the alum and TT-primary granulomas over the 28-day experimental period. In contrast, TT-secondary granulomas from Dact-treated mice showed an initially delayed response, but by Day 7 and thereafter, values similar to the nondrug-treated group were attained.

5. Plasma cell response. Plasma cells were present in all three types of granulomas in mice not treated with Dact. Alum granulomas from Dact-treated mice contained no plasma cells during the 28-day experimental period. Dact affected TT-primary granulomas by significantly inhibiting the number of plasma cells on Day 14 ($p < 0.05$); in this group, a few plasma cells were noted on Day 28. In the TT-secondary granuloma, Dact delayed plasma cell formation.

The average rate of production of plasma cells in TT-secondary granulomas was de-

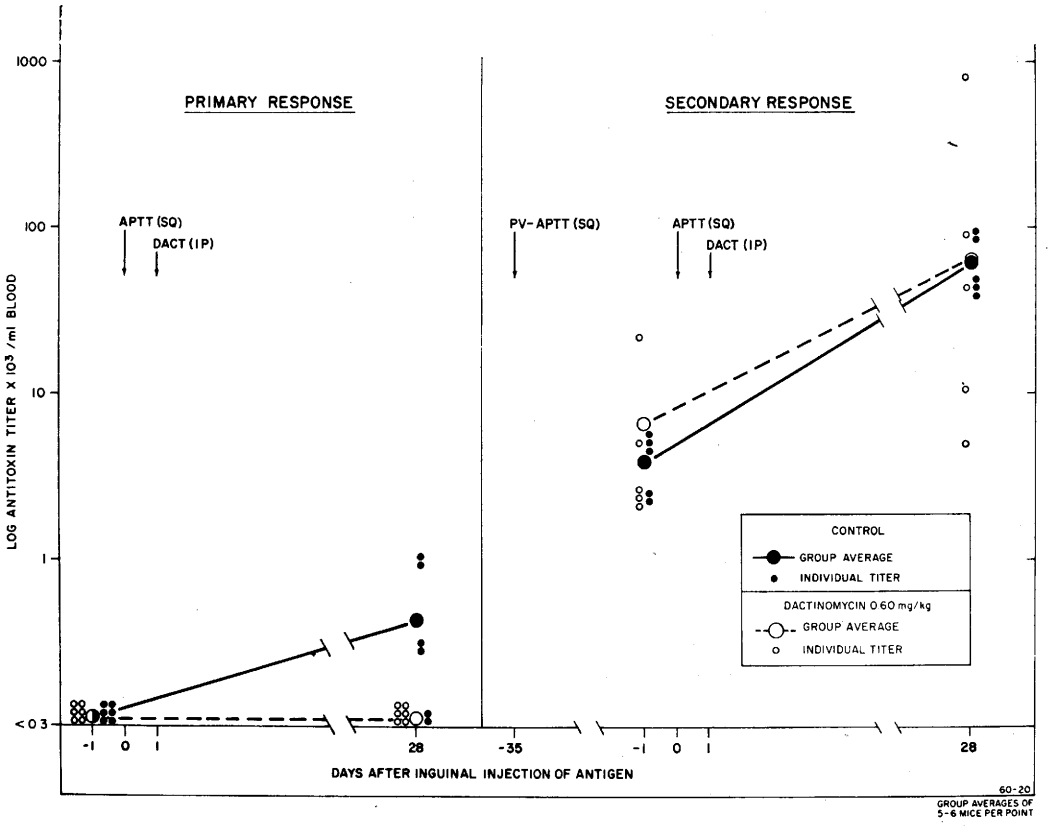

FIG. 4. Effect of Dactinomycin on antitoxin titer in blood during the primary and secondary responses to tetanus toxoid.

rived from the slopes of the lines connecting the responses on two successive autopsy days. Between Days 7 and 14, the rate of production in HBSS-treated mice was 8.5 times greater than in Dact-treated mice. Between Days 14 and 28, Dact-treated mice recovered their capacity to respond; plasma cells were produced at a rate of 2100 per day in HBSS-treated mice and 2800 per day in Dact-treated mice.

II. Levels of circulating antitoxin after inguinal injection (Fig. 4). A. Primary immune response. A single ip injection of Dact inhibited antitoxin producion during the 28-day experimental period. Mice not receiving Dact had a detectable rise in antitoxin levels by Day 28.

B. Secondary immune response. Treatment with Dact did not significantly alter the capacity of primed mice to mount a secondary antitoxin response to challenge. By Day 28, the same antitoxin levels were present in both Dact and HBSS-treated mice.

Discussion. This study corroborates previous findings that Dactinomycin can affect granuloma formation (7–9) and reports the additional observation that quantitative cellular differences are evident depending upon the type of granuloma induced.

Dactinomycin is shown to have a different effect on the two granulocytic cell types present in the granulomas (Fig. 2). During most of the experimental period, neutrophil responses were depressed in the alum and in the TT-primary granulomas, whereas in the TT-secondary granulomas, their response was similar to control values. Neutrophils have been shown to be chemotactic to antigen–antibody complexes (10) which would be present in the TT-secondary granulomas since measureable levels of antitoxin were demonstrated in the blood (Fig. 4). The

neutrophil response in alum and TT-primary granulomas implies chemotaxis due to other Dactinomycin-sensitive mechanisms. Antigen–antibody complexes must not be involved since antibody is not present during the initiation of these granulomas. The eosinophil response does not appear to be Dactinomycin sensitive. Moreover, the eosinophil response is independent of circulating antitoxin since the number of eosinophils in drug-treated animals is not significantly depressed in the TT-primary granulomas when there is no detectable antitoxin level. This confirms previous studies indicating that antigen–antibody complexes are not the sole cause of the eosinophil response (11–13). Different mechanisms for the accumulation of neutrophils and eosinophils seem evident, and this has been proposed by Keller and Sorkin (14).

Dactinomycin has been shown to depress the number of circulating lymphocytes and monocytes as well as the cell content of hemopoietic tissue (4). This is reflected in both the alum and TT-primary granulomas, which reveal a depressed mononuclear cell response over the 28-day experimental period. However, in the TT-secondary granulomas, no significant effect on the mononuclear cell response was observed, which may be due in part to memory cells known to be present in the secondary granulomas (1). Some of the memory cells formed during a primary response are recirculating cells (15) and under the influence of an inflammatory stimulus, these cells enter the granuloma and react with antigen. Further recruitment of cells by the various mediators of inflammation (16) apparently supersedes the metabolic or toxic effects of the drug.

It is well established that Dactinomycin modifies protein synthesis by inhibiting the production of DNA-dependent RNA (17). This relates to the significant decrease in all groups on Days 2 and 4 in the number of basophilic mononuclear cells. These cells are actively protein-synthesizing cells inasmuch as cytoplasmic basophilia is indicative of ribosomal RNA involved in protein synthesis (Fig. 3). At least two types of protein-secreting cells were present in the granulomas—fibroblasts and plasma cells. The accumulation of fibroblasts with associated collagen formation has been shown to occur during the first week after granuloma induction with APTT (1). Therefore, the basophilic mononuclear cells present during the first 4 days may be primarily fibroblasts synthesizing collagen. After Day 4, both TT-primary and TT-secondary granulomas from mice not treated with Dactinomycin contain an increased basophilic mononuclear cell population. The associated increase in the number of plasma cells beginning on or about Day 14 may have been due to a transformation of some of the basophilic mononuclear cells to plasma cells. Dactinomycin depresses this sequence in the alum and TT-primary granulomas and delays it in the TT-secondary granulomas (Fig. 2). Plasma cell production in TT-secondary granulomas of drug-treated mice was delayed for two weeks, then increased at the same rate as in mice not treated with the drug. It appears that Dactinomycin has only a delaying effect on the rate of plasma cell formation during the anamnestic response. This corresponds with its failure to inhibit circulating levels of antitoxin in the secondary immune response (Fig. 4).

It would appear that quantitative cell analysis of granuloma formation could prove useful in studying and evaluating the action of anti-inflammatory and immunosuppressive agents upon inflammatory and immunocompetent cells as they respond to pathogenic agents.

Summary. The data from this study indicates that Dactinomycin affects the number and type of cells involved in granuloma formation. Neutrophils and mononuclear cells were decreased in alum and TT-primary granulomas, but no such effect was noted in TT-secondary granulomas. In contrast, the eosinophil response in all three types of granulomas appeared to be unaffected by Dactinomycin treatment. The number of plasma cells present and the serum antitoxin titers obtained in the primary response were both significantly depressed. In the TT-secondary granulomas, the rate of plasma cell formation, although initially delayed, was unaffected. It is suggested that the granuloma

is a reliable index of the host's capacity to react to antigen and can serve as a model to study the effects of anti-inflammatory and immunosuppressive agents on such reactions.

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