# A Quantitative Procedure for the Study of Cells in Experimentally Induced Granulomas[1] (36305)

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Granuloma formation has been the concern of many investigators at both the research and clinical levels. The procedures used to study granulomatous inflammation have included morphological observation (1), analysis of cell migration patterns (2–4), and measurement of granuloma size in terms of granuloma diameter (5), volume (6), and weight (7). These methods, however, lack the capacity to quantitate the actual number of each cell type involved in the formation of a granuloma.

Our laboratory has been particularly concerned with quantitation procedures for the study of the inflammatory exudate in the peritoneal cavity (8). In the course of these studies, it was noted that granulomatous tissue was subsequently formed at the site of inflammation (9, 10). Our investigations were then extended to determine if it would be possible to develop a technique for quantitating the cells in the granuloma. This study describes a procedure for dispersing experimentally induced granulomas into their cellular components. Also presented are the results obtained when this procedure was applied at different stages of granuloma formation following the injection of antigenic and nonantigenic substances.

*Materials and Methods. Induction of granulomas.* In this investigation, 3 types of granulomas were induced by subcutaneous injections into the inguinal region of adult female $BDF_1$ hybrid mice. Type I (primary granuloma) consisted of granulomas formed in response to a priming injection of 0.4 ml of tetanus toxoid adsorbed onto aluminum phosphate (APTT) (Parke Davis). Type II (secondary granuloma) consisted of granulomas formed in response to a challenging injection of 0.4 ml of APTT in mice which had been primed subcutaneously in the dorsal neck 5 weeks previously with 0.2 ml of APTT mixed with 0.2 ml of pertussis vaccine (PV) (Eli Lilly) adjuvant diluted 1:10 with saline. Type III (alum granuloma) consisted of granulomas formed in response to an injection of 0.4 ml of a nonantigenic substance, aluminum phosphate (AP), (5 mg/ml of saline) into mice which had been primed with PV-APTT as above. In order to facilitate location of the granulomas, activated sterile carbon (C) (Merck and Co.) was mixed with AP, APTT and PV-APTT solutions in a concentration of 2 mg/ml.

*Processing of granulomas.* Mice were killed by cervical dislocation, dipped quickly into a solution of pHisoHex (Winthrop Laboratory, New York), and the skin was peeled back to reveal the subcutaneous granulomas. Each granuloma was freed of any adherent connective tissue and then subjected to dispersion and digestion procedures in a siliconized tube containing 1 ml of a cold, freshly prepared solution of collagenase.

Collagenase, from *Clostridium histolyticum* (General Biochemical Corp.) was prepared at a concentration of 4 mg of collagenase/ml of Hanks' balanced salt solution (pH 7.1–7.2). After 1–3 hr of incubation at 37°, the stroma dissolved; and the granuloma fell

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apart upon shaking. Intermittent shaking of the tubes was essential for further disruption of the granulomas. Granulomas from primed mice took longer to break up than those from unprimed mice. Pronase, prepared from *Streptomyces griseus,* (1 mg/0.1 ml) (Grade B, Calbiochem, Los Angeles, CA) was then added to the incubating suspension to produce more complete dispersion of the cells. The addition of pronase also helped eliminate debris which would otherwise interfere with cell quantitation.

One-half hour after the addition of pronase, incubation was stopped by placing the tubes on a bed of crushed ice. Two samples were removed from each tube (with separate white blood cell pipettes) and diluted 1:20 with Carpentier's solution (11). Total and eosinophil counts were made using Speirs-Levy eosinophil counting chambers (C. A. Hausser, Philadelphia, PA). The remainder of the suspension in each tube was transferred to a 3 ml silicone coated conical centrifuge tube. Decomplemented fetal bovine serum (0.25 ml) diluted 1:1 with distilled water was added to the bottom of each centrifuge tube and the tubes were spun for 5 min at 500 rpm in a refrigerated centrifuge. The supernatant fluid was discarded and a sample of the cellular pellet remaining at the bottom of the tube was spread with a No. 0 fine sable artist's brush (12) onto gel coated slides, and subsequently stained with May–Grüenwald Giemsa blood stain (8). Differential cell counts were made from the smear. By multiplying the percentage of each cell type by the total cell count, the total number of each cell type in the granuloma was estimated. An index of the reliability of the counts was obtained by comparing the number of eosinophils in the counting chamber with the number obtained in the differential count.

In order to determine its effectiveness, the quantitative technique was used to assay responses to foreign materials at specific intervals over a period of 28 days. Six to eight mice for each of the 3 types of granulomas induced were sacrificed on days 1, 4, 7, 14, and 28 after the inguinal injection. Granulomas developing in that region were removed, the number of component cells was estimated for each granuloma, and the averages were obtained for each of the three groups.

The cells were classified as granulocytes (neutrophils, eosinophils, and mast cells) and agranulocytes. The agranulocytes included (i) mononuclear cells (fibroblasts, macrophages, and lymphocytes); (ii) basophilic mononuclear cells (dark blue staining mononuclear cells of small, medium, and large size); (iii) plasma cells; and (iv) giant cells (large binucleated and occasionally trinucleated cells). Mast cells and giant cells collectively accounted for relatively few of the constituent cells in all subcutaneous granulomas studied and were not graphically represented.

For histopathological correlation, the granulomas were examined independently by another observer using methods previously described (10), and treated as unknowns. The eosinophil leukocyte infiltration within the granulomas was estimated and recorded since this cell was quite readily identified and was the least morphologically ambiguous appearing cell.

*Results. I. Quantitative cell responses utilizing dispersion procedure. Total cell response.* The total number of cells present during the development of the granulomas was highest on all days tested in primed mice reinjected with specific antigen, rising to 4 million cells by day 7 and remaining at that level throughout the experimental period (Fig. 1). The response in unprimed mice showed a similar pattern but was of a lower magnitude. The response to the nonantigenic aluminum phosphate showed a different pattern and a lower response on days 7 and 14 from that of the other two groups.

The number of each of the most common cells found in the 3 types of granulomas is shown in Fig. 2.

*Neutrophil response.* In all granulomas, the neutrophil response rose soon after granuloma induction, decreased after day 1, and remained at a low level throughout the experimental period.

*Eosinophil response.* The number of eosinophils present in the secondary granulomas

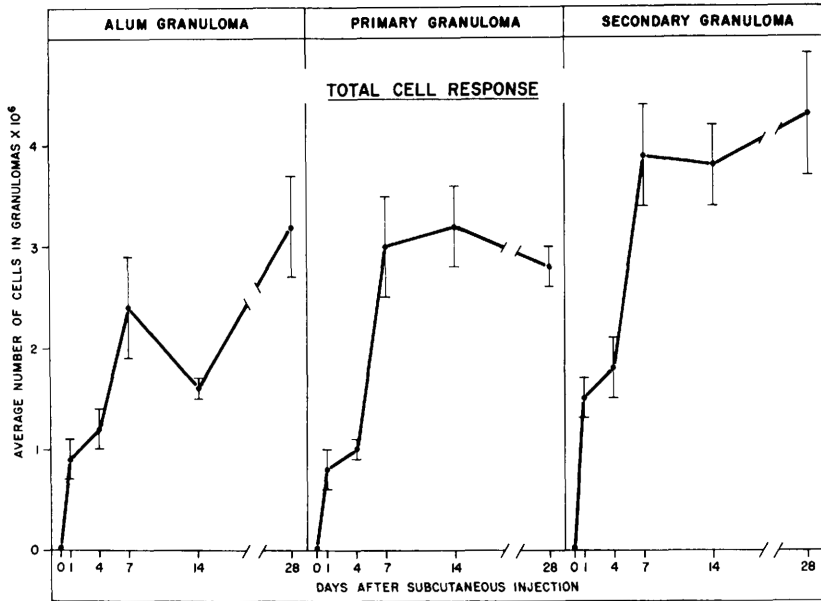


FIG. 1. The total number of cells present in alum, primary, and secondary granulomas on specific days after induction subcutaneously in the inguinal region: Each point represents 6–8 animals ± the standard error of the mean.

was considerably greater than in the other groups. They reached a peak of 800,000 on day 7, and remained elevated throughout the experimental period. An eosinophil response was obtained in unprimed mice but it was of a lower magnitude reaching a peak of 550, 000 cells at day 14. The number of eosinophils in alum granulomas remained relatively low throughout the experiment.

*Mononuclear cell response.* A wide variety of mononuclear cells was observed on cell smears in terms of cell size and shape, nuclear position, and degree of cytoplasmic basophilia. It became necessary to classify these cells into nonbasophilic and basophilic mononuclear cells. The nonbasophilic mononuclear cells referred to as "mononuclear" cells included fibroblasts, macrophages, and lymphocytes. These were most prominent in the later stages of all granulomas studied. Somewhat higher responses were obtained in secondary granulomas, showing a range of 1 million cells after day 1, to 3.5 million cells by day 28.

*Basophilic mononuclear cell response.* Basophilic mononuclear cells, varying in size and degree of cytoplasmic basophilia, have been noted on cell smears from lymph nodes of mice responding to sheep red blood cells (13). Smears of granuloma cells in this study revealed a population of basophilic mononuclear cells also showing a range in cell size and cytoplasmic basophilia. All members of this population of cells have been reported here collectively as the basophilic mononuclear cell response. These cells were present in greatest number in secondary granulomas, peaking between days 4 and 7. Primary and alum granulomas had fewer cells at these times.

*Plasma cell response.* Cells were classified as plasma cells if they met the following requirements: a cytoplasmic basophilia, a negative Golgi region, an eccentrically situated nucleus with peripheral clumping of the chromatin. Strict adherence to these criteria was followed; and cells not meeting them were classified as either basophilic or nonbasophilic mononuclear cells.

Plasma cells were present in all granulomas. However, they were most conspicuous in secondary granulomas. Their number in-

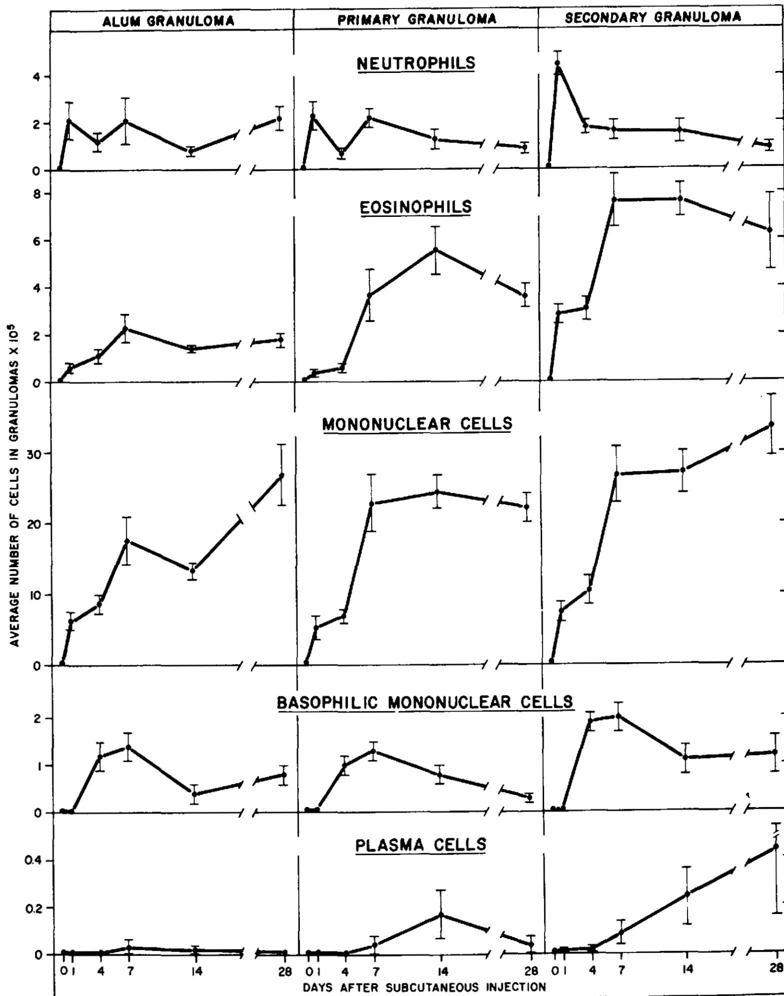


FIG. 2. The total number of each cell type present in alum, primary, and secondary granulomas on specific days after induction subcutaneously in the inguinal region: Each point represents 6–8 animals ± the standard error of the mean.

creased after day 4 in this group, reaching a level of 45,000 by day 28. The plasma cell response was lower in primary granulomas and still lower in alum granulomas, averaging approximately 200 cells on day 28. The linear regression equations between days 4 and 7, 7 and 14, and 14 and 28 were derived from the secondary granulomas and the slopes of these lines were determined. No statistical difference was found between these slopes which indicated that the rate of change of plasma cells in the secondary granuloma between days 4 and 28 was constant. From this, it was determined that the plasma cells increased during this period at the rate of about 2000 cells/day.

*II. Estimate of cellular responses utilizing conventional histopathological procedures.* The results by histopathological observation and the results by the quantitative dispersion technique are summarized and compared in Table I.

It can be noted that in those groups of animals where the eosinophil leukocyte infiltration was substantially high, both methods indicated this for the most part.

TABLE I. Comparison of Eosinophil Leukocyte Infiltration Within the Aggregates (granulomas) Obtained by Conventional Histopathological Methods[a] with That Obtained by the Quantitative Technique.

| Granuloma type | Method of analysis (technique) | Day: 1 | 4 | 7 | 14 | 28 |
|---|---|---|---|---|---|---|
| Alum | Conventional | <+ | <+ | <+ | <+ | <+ |
| | Quantitative | 5% | 8% | 9% | 9% | 6% |
| Primary | Conventional | <+ | <+ | + | + | <+ |
| | Quantitative | 5% | 7% | 11% | 16% | 13% |
| Secondary | Conventional | + | 2+ | 2+ | 2+ | + |
| | Quantitative | 19% | 18% | 20% | 21% | 13% |

[a] In approximating the intensity of eosinophil leukocyte infiltration an arbitrary scale was utilized: 2+ = approximately 20–40% of the infiltrating cells; + = approximately 10% of the infiltrating cells; and <+ = less than 10% of the infiltrating cells per random section of granuloma.

*Discussion.* A technique was described for dispersing granulomas into their component cells and estimating both the total cell population and the number of each different cell type present. A quantitative study was made of the cells present at different times during granuloma development. The procedure was sufficiently sensitive to detect variations in the component cell population depending upon the age of the granuloma, the immunological state of the organism, and the type of substance (antigenic or nonantigenic) used to induce the granuloma. In other experiments (14) the cells obtained from the granulomas were found to be viable when cultured *in vitro* after exposure to proteolytic enzymes. Chemotaxis, adhesion to glass, and phagocytosis were demonstrated by phase microscopy.

The results of the dispersion and digestion technique were compared independently with the results obtained by utilizing conventional histologic procedures. It was not possible by conventional histology to estimate accurately the total number of cells, or the absolute number of each cell type, within a granuloma. Nevertheless, it was possible to make certain judgments regarding the relative proportions of the infiltrating cells. A good correlation was found between the two procedures. That is, in those granulomas where utilizing the dispersion and digestion technique large numbers of eosinophils were found, inspection by conventional histology of random samples of comparable granulomas also revealed a high proportion of these cells. This correlation suggests that, with this new technique, accurate estimations of the proportions of infiltrating cells can be made.

It is felt that with this procedure, the experimentally induced granuloma can be used as a model to study the types of cells responding to a particular foreign material. It may also be used to determine the capacity of cells to respond to a particular stimulus under various experimental and disease conditions. For example, the number of each cell type in an induced granuloma may be a possible index of the capacity of that particular cell type to respond to a specific stimulus. In addition, the procedure may be of value in the study of granulomas obtained clinically at biopsy (15) since different etiological agents will induce different cellular responses depending upon the nature of the agent and the sensitivity of the host.

*Summary.* A procedure for a quantitative cell analysis involving enzymatic digestion of granulomatous stroma was described. The results of its ability to differentiate between granulomas induced by antigenic and nonantigenic material and granulomas from primed and unprimed mice were presented. Correlation with conventional histopathological methods was shown. The use of this procedure for studying cells reacting to foreign material was discussed.

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