A Quantitative Approach to the Study of Inflammatory Cells.* (26300)

R. S. Speirs, E. E. Speirs and V. Jansen
Department of Anatomy, State University of New York, Downstate Medical Center, Brooklyn, N. Y.

The presence of antigenic material within an organism produces not only localized inflammatory responses but also changes in number and types of cells found in regional lymph nodes, spleen and hemopoietic tissues (1,2). Antibody is formed and the organism altered in such a manner that future reexposure to the same antigen produces different magnitudes of cellular and humoral responses (3.4). To achieve an understanding of the origin, function and fate of the cells involved in these responses, technics for enumeration of cells migrating into an inflammatory area, as well as those leaving the area, must be applied. This can be accomplished by inducing the inflammation in the peritoneal cavity where routine hematological technics can be applied to determine amount of free fluid and total number of each cell type suspended in the fluid(5,6). This paper outlines a procedure for quantitative estimation of cellular changes in the inflammatory exudate following intraperitoneal injection of an antigen into sensitized and non-sensitized mice.

Materials and methods. Eighty C57 BL/6 mice, 3 to 8 months of age, were used in these experiments. One half of the animals were sensitized (immunized) by a series of 7 subcutaneous injections of 5 Lf Diphtheria toxoid (Lederle). Thirty days after the last sensitizing injection, 30 mice from each group were injected intraperitoneally with 5 Lf of Diphtheria toxoid in 0.4 ml isotonic saline. The remaining mice were used as uninjected controls. Daily autopsies were performed on 3 animals from each injected group and on one from each of the uninjected groups.

Sufficient fluid is usually present in the peritoneal cavity of mice to permit total cell counts and to make smears for differential counts. However, in the experiments reported here, an injection of 0.2 ml of fluid was made immediately prior to autopsy to assist in washing out the cells and to assure that a sufficient number of cells would be present to perform not only quantitative counts, but also to permit *in vitro* metabolic studies. The cellular responses obtained with diluted peritoneal fluid aspirate corresponded with data obtained in other experiments in which the peritoneal fluid was not diluted at time of autopsy.

The autopsies were performed as follows: The animals were weighed and given an intraperitoneal injection of 0.2 ml of fluid consisting of equal parts of Hank's salt solution and polyvinylpyrrolidone (Schenley). They were then immediately killed by severing the cervical spinal cord. The inflammatory cells were aspirated from the peritoneal cavity, weighed, and samples taken for total counts, eosinophil chamber counts and for differential cell counts. Carpentier's solution† was used as a diluent since it enables both total cell and eosinophil cell counts to be performed. Counts were made in Speirs-Levy Eosinophil chambers, (an average of 370 cells per animal). The slides for differential counts were stained for 3½ minutes in May-Gruenwald Blood stain after methyl alcohol fixation. Differential cell counts were estimated from a minimum of 500 cells per experimental animal.

Results. Fluid and cell determinations obtained at various times after an intraperitoneal injection of diphtheria toxoid into sensitized and non-sensitized mice are averaged and illustrated in Fig. 1-4. The greatest amount of fluid in both groups was found in animals autopsied 24 hours following intraperitoneal injection, when the amount of fluid in the sensitized mice was approximately

* These data were presented at Federation Meetings, Chicago, Ill., April 1960. This work was supported by research grants from Nat. Inst. Health, P.H.S.

† Formula for Carpentier's solution(7): 1 ml eosin Y (2% aqueous solution), 3 ml neutralized formalin, 96 ml distilled water.

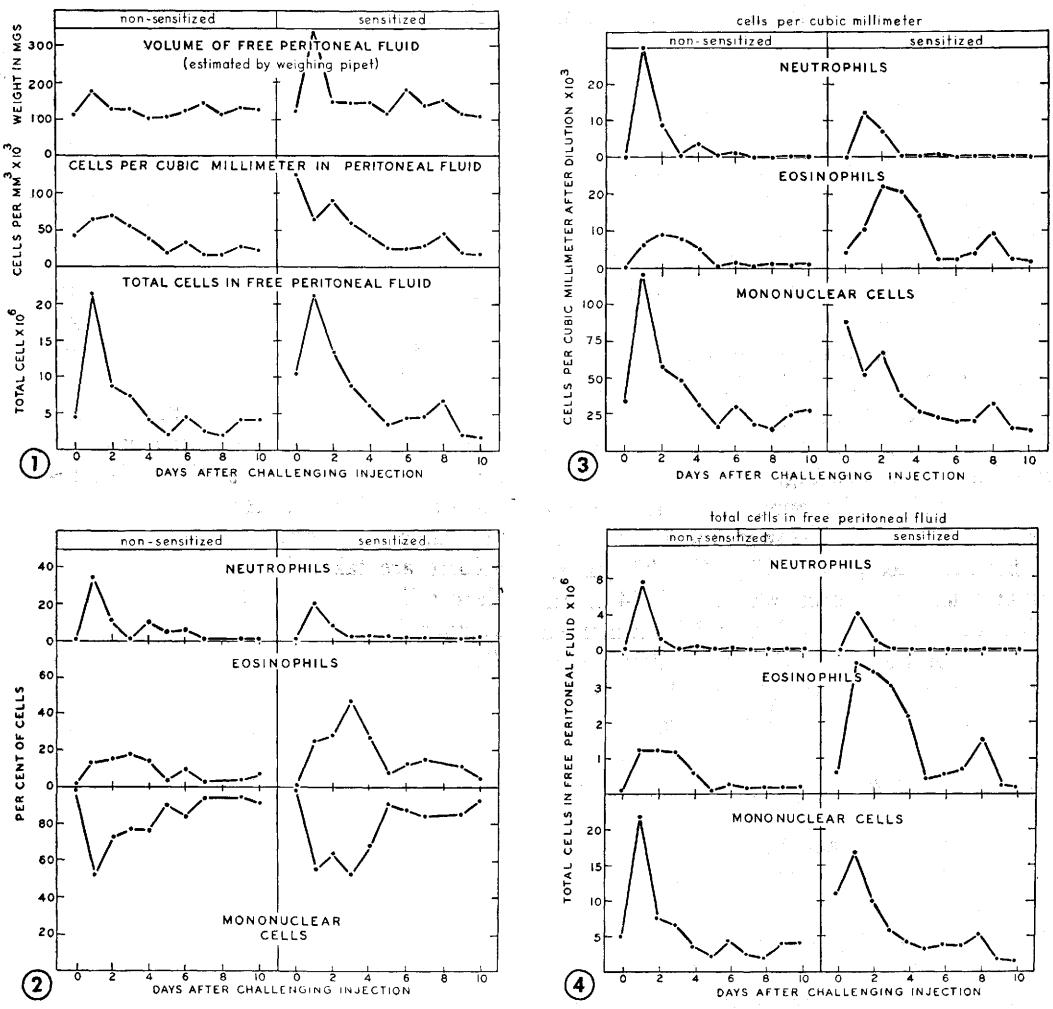

FIG. 1. Fluid volume and cellular changes in peritoneal exudate following intraper. inj. of diphtheria toxoid into sensitized and non-sensitized mice.

FIG. 2. Qualitative cellular response following intraper. inj. of diphtheria toxoid.

FIG. 3. Quantitative cellular response following intraper. inj. of diphtheria toxoid (cells per cu mm³).

FIG. 4. Quantitative cellular response following intraper. inj. of diphtheria toxoid (total numbers of each cell type which could be aspirated from peritoneal cavity).

twice that which could be obtained at other times (Fig. 1).

Number of cells per cubic millimeter of aspirated fluid is also shown in Fig. 1. Total cell estimate was obtained by multiplying number of cells per cubic millimeter by volume, assuming that 1 cmm was equivalent to 1 mg. The greatest number of cells which could be aspirated from the peritoneal cavity was found on the first day after antigen injection.

Fig. 2 illustrates the types of cells found in the peritoneal exudate. Fluid taken from the non-inflamed peritoneal cavity (0 day) consisted almost entirely of mononuclear cells (96%), the remainder being eosinophils, neutrophils and mast cells. In earlier experiments the mast cells did not show significant changes in number and they were not recorded at this time(4,5,6).

Injections of diphtheria toxoid produced rapid changes in cellular population of the peritoneal cavity which tended to persist throughout the 10-day period. At 24 hours,

number of neutrophils had increased from less than 1% to 34% in the non-immunized mice and to 20% in the immunized mice. During the first 7 days both percent and total number of neutrophils were higher in the non-immunized animals than in the immunized mice. On the other hand, the eosinophil response was consistently higher in the immunized mice. Within 24 hours after antigen injection the number of eosinophils which could be removed from the peritoneal cavity of immunized mice increased from 58,000 to 3.6 million. By the third day, the eosinophils comprised 47% of all the cells found in the peritoneal cavity of the immunized animals. See Fig. 3.

The number of mononuclear cells in the peritoneal aspirate followed the same curve in both injected groups (Fig. 4). The maximum number of cells was found 24 hours after antigen injection, followed by a marked decrease between the 1st and 3rd days. Phagocytized material was commonly observed in the mononuclear cells. During the first day numerous vacuoles containing necrotic neutrophils as well as antigenic material were seen. Between the 3rd and 8th days, eosinophils in various stages of degeneration were noted within the macrophages of the sensitized animals. Morphological differences in the mononuclear cells were also apparent, beginning on 4th day of inflammation. Cells with intense cytoplasmic basophilia as well as plasma cells were seen, particularly in the sensitized animals. However, since transitional forms made clear-cut classification difficult, these cells were all grouped as mononuclear cells.

Discussion. The results presented here indicate that the cells taking part in an inflammatory response can be conveniently studied in the peritoneal cavity. Routine hematological technics can be applied at various times after initiation of the inflammation and a sufficient number of cells removed to study both qualitative and quantitative changes.

Comparisons were made of different methods of enumerating the inflammatory cells. Qualitative data, expressed as percent (Fig. 2) can be compared with quantitative data, expressed as cells per cmm (Fig. 3) or total cells in the aspirate (Fig. 4). The neutrophil counts appear similar regardless of the method used for presenting the data. On the other hand, comparison of the eosinophil counts indicates marked differences. The increase in fluid volume, as well as the numerical increase in other inflammatory cells, tends to mask the eosinophil response when qualitative data alone are considered.

Mononuclear cells make up a heterogeneous group comprising approximately 96% of all cells normally found in the uninflamed peritoneal fluid. Following an injection of any inflammatory material, the granulocytes migrate into the peritoneal cavity and therefore markedly decrease the proportion of these cells (Fig. 1). However, when the cells per cubic millimeter and the increased fluid volume are taken into account the mononuclear cells are found to greatly increase in number (Fig. 2,3) during the first 24 hours. This would be impossible to determine from the qualitative data alone (differential counts).

Although both granulocytes and mononuclear cells were present in maximum numbers on the first day after injection, there were distinct differences in rate of disappearance of each cell type on subsequent days. Neutrophils decreased in number very rapidly and were seldom seen after the 2nd day. On the other hand, eosinophils tended to persist for longer periods, especially in the immunized animals. The mononuclear cells decreased in number between the 1st and 3rd days but throughout the whole period remained the predominant cell type in the inflammatory exudate.

The factors involved in disappearance of inflammatory cells vary, depending upon the cell type. Neutrophils are phagocytized by mononuclear macrophages, and the remains of these cells were found in macrophages within 24 hours of onset of inflammation. Eosinophils are also phagocytized by macrophages, but usually between the 3rd and 8th day of inflammation. Although phagocytosis of injured mononuclear cells may occur, many of the cells appear to be viable and are carried out of the inflammatory area into the lymphatic and blood vessels by the ede-

metous fluids(2). Thus, rate of phagocytosis of the granulocytes, as well as rate of migration of the mononuclear cells from the inflammatory area, account to a great extent for the sequence of cell types seen in the inflammatory exudate.

The number of each cell type responding to an injection of antigen varies depending, in part, upon sensitivity of the animal to that antigen. The neutrophil response appears to be somewhat greater in animals injected with antigen for the first time than in reinjected animals. In contrast to neutrophils, eosinophils are found in greater numbers when the antigen is reinjected. The local increase in eosinophils is a consistent finding whenever an antigen is repeatedly injected into an animal(4). This has recently been confirmed by Litt(11) who observed a quantitative local eosinophilia in the guinea pig following repeated intraperitoneal injections of an antigen. Archer(12) has also found local increases in eosinophils at the site of reinjection of antigen in the skin of the horse. The relation of these cells to adrenal cortical hormone secretions and to antibody formation has been presented earlier(8,9,10).

The mononuclear cells of the sensitized animals showed marked changes in morphological appearance during various periods of the inflammation. Intense basophilic staining of the cytoplasm was observed between the 4th and 10th days of inflammation. It is considered significant that these changes occur in macrophages which have engulfed eosinophils(2,3).

Summary. This paper compares procedures for qualitatively and quantitatively studying cellular responses to inflammatory substances. It was noted that estimates of total cells in the aspirated exudate suggested conclusions not obvious from data obtained from differential counts. These data suggest that the observed sequence of inflammatory cells is due not only to the initial migration of cells into the inflamed area, but to the rate of phagocytosis of the granulocytes by the macrophages. The inflammatory exudate of sensitized animals contained fewer neutrophils and greater numbers of eosinophils. Plasma cells and mononuclear cells with intense basophilic cytoplasm were frequently observed following the disappearance of eosinophils.

1. Harris, H., *Physiol. Rev.*, 1954, v34, 529.
2. Speirs, R. S., *Reticulo-Endothelial Structure and Function*, Chap. 7, 1960. Ed. by J. H. Heller, Ronald Press, N. Y.
3. ———, *Ann. N. Y. Acad. Sci.*, 1958, v73, 283.
4. ———, *ibid.*, 1955, v59, 706.
5. Speirs, R. S., Dreisbach, M. E., *Blood*, 1956, v11, 44.
6. Speirs, R. S., Wenck, U., Dreisbach, M. E., *ibid.*, 1956, v11, 56.
7. Carpentier, P. J., *Nature*, 1955, v175, 1128.
8. Speirs, R. S., *ibid.*, 1958, v181, 681.
9. Dreisbach, M. E., Snell, G. D., Speirs, R. S., *J. Nat. Cancer Inst.*, 1956, v17, 297.
10. Speirs, R. S., Wenck, U., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 571.
11. Litt, M., *Blood*, 1960, v16, 1318.
12. Archer, R., personal communication.

Received July 13, 1960. P.S.E.B.M., 1961, v106.

DNA Synthesis in Inflammatory Cells of Immunized and Non-Immunized Mice.* (26301)

R. S. SPEIRS, Y. OSADA† AND V. JANSEN
Department of Anatomy, State University of New York, Downstate Medical Center, Brooklyn, N. Y.

This study utilizes quantitative technics developed earlier(1) to determine the number of cells synthesizing DNA in the inflam-

* This work was supported by research grants from Nat. Inst. Health, PHS, and from Atomic Energy Comm. The experiments reported were carried out in part by students attending a course of Experimental Hematology.

† Recipient of a Research Fellowship from Rockefeller Foundation. Present address: Inst. of Public Health, Tokyo, Japan.