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The Neutrophil Response to Polyvinyl Sponge Implantation[1] (41523)

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Abstract. Neutrophil release and migration in mice were studied over a 24-hr period after the sc implantation of a single polyvinyl sponge. The release of neutrophils from the marrow was evaluated by directly counting the residual neutrophils in the femoral marrow of animals with sponges. Sponge and tissue neutrophil content was determined by extraction and assay of myeloperoxidase (MPO), a marker enzyme for neutrophils. A maximum depletion of 48% of the mature neutrophils in the marrow was observed 5 hr after sponge implantation, in keeping with significant release of neutrophils for migration to the sponge. The released cells were not found in the circulating granulocyte pool, since neutropenia was noted. The accumulation of neutrophils in the sponge increased throughout the 24-hr period, whereas in the tissue adjacent to the sponge maximum accumulation of neutrophils occurred within 7 hr. In fact, neutrophils migrated to at least three sites—the sponge, the skin overlying the sponge, and the skin in which an incision had been made to insert the sponge. The sponge content of neutrophils represented 0.3–33% of the neutrophils migrating to the combined lesion (sponge and skin sites). Therefore, if the neutrophil response to foreign body implantation is to be measured in its entirety, it is necessary to quantify not only the neutrophils within the foreign body but also those in the tissues surrounding it. These studies describe an animal model for neutrophil release and migration to tissues following a standard stimulus. It is proposed that this model may be useful in exploring the factors which influence the release and migration of neutrophils *in vivo.*

During the acute inflammatory process, neutrophils migrate to and accumulate in the tissues. This neutrophil response constitutes an important factor in host resistance to infection and may also play a significant role in some inflammatory diseases (1). In order to better understand neutrophil locomotion many investigators have examined the movement of neutrophils *in vitro* (2, 3). From such studies, valuable information has been gained regarding the physiology of neutrophil chemotaxis. However, *in vitro* studies do not address at least two events observed *in vivo* i.e., the release of stored neutrophils from the marrow into the blood, and their subsequent migration to sites of inflammation in the tissues. Thus, we reasoned that the *in vivo* study of neutrophil movement out of the marrow and then into tissue lesions may be useful to more completely understand the neutrophil inflammatory response in intact animals and man.

Several investigators have studied the accumulation of neutrophils in the vicinity of inflammatory lesions by enumerating the neutrophils in exudates (4, 5). The study of neutrophils accumulating in implanted sponges has yielded important data regarding the effect of various pharmacologic agents upon neutrophil movement *in vivo* (4, 6, 7). However, such investigations have concentrated upon the measurement of neutrophils within the sponges. In the present experiments our goal was to use the stimulus of an implanted sponge to evaluate the release of neutrophils from the marrow, their entry into the blood, and the accumulation of neutrophils in not only the sponge but also the tissue surrounding it. We quantified marrow cellularity directly, determined the blood neutrophil concentrations, and employed a new method for measuring the sponge and tissue content of neutrophils using the enzyme myeloperoxidase as a marker for neutrophils (8). We propose that the standard-

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ized inflammatory response thus defined in intact animals will be useful in understanding the factors which influence the release of neutrophils from the marrow and their migration to tissues *in vivo.*

Materials and Methods. *Polyvinyl sponge implantation.* Eight mg polyvinyl sponge discs (Unipoint Industries, Inc., High Point, N.C.) were boiled for 1 hr in water which had been subjected to charcoal filtration and deionization by a Milli-Q filter system (Millipore Corp., Bedford, Mass.). Then, the sponge discs were placed into surgically created sc pockets on the shaved dorsum of 5-week-old (18–22 g) Swiss–Webster female mice (Simonsen Labs) anesthetized with methoxyfluorane. Pockets were closed with a single 4/0 silk suture and sealed with collodion (U.S.P., Robinson Labs, Inc., San Francisco, Calif.). To ensure uniformity, control mice were also anesthetized for study concurrently with experimental groups.

Quantitative studies of circulating and bone marrow neutrophils. Mice were anesthetized with methoxyfluorane, and then blood was drawn from the inferior cava into a syringe containing EDTA. Nucleated blood counts were performed electronically (Coulter Electronics, Hialeah, Fla.). Coverslip blood smears were stained with Wright's stain and a 100-cell differential was done. For quantitative bone marrow studies, both femurs were removed and flushed three times with 2 ml Hanks' balanced salt solution (HBSS) using a 25-gauge needle inserted into the femoral shaft. The cells were counted electronically, and a 500-cell differential count was done on coverslip marrow smears. The femoral neutrophil storage pool (NSP) was defined as all mature neutrophils (PMN + band + metamyelocytes). The total body NSP was calculated by multiplying the femoral NSP by 8.5, since it has been shown that the content of combined femurs is 11.8% of the total marrow of the mouse (9).

Quantification of neutrophils migrating to the sponge. Sponges were removed and wrung out 20 times in 0.5 ml HBSS. The cells were counted electronically and a 100-cell differential count was performed. The number of neutrophils contained in the sponge was calculated from the fluid volume, the cell, and the differential count.

Preparation of tissue and sponge specimens for myeloperoxidase (MPO) assay. Tissue specimens were minced with scissors in 2 ml 0.5% hexadecyl trimethylammonium bromide (HTAB) in 50 m*M* potassium phosphate buffer, pH 6.0. The suspension was sonified (Heat Systems—Ultrasonics, Plainview, N.Y.) in an ice bath and frozen. The specimens were thawed and frozen three times and then homogenized for 10 min in an ice bath. The homogenized suspension was again sonified and reserved at −80°.

Sponges for MPO assay were placed in 1 ml 0.5% HTAB and then sonified and frozen. The solution with sponge in it was frozen and thawed three times, and the sponges were wrung out and removed. The remaining suspension was subjected to sonication, frozen, and reserved for later MPO assay.

Preparation of neutrophils from the blood and sponge for MPO assay. Leukocytes were separated from the pooled blood of 10–15 mice in discontinuous gradients of Ficoll (Pharmacia Fine Chemicals, Piscataway, N.J.) and sodium diatrizoate (Winthrop Lab, N.Y.) (10, 11). Contaminating erythrocytes were subjected to hypotonic lysis, and the resulting WBC pellet was washed in HBSS and counted electronically. Two hundred-cell differential counts were performed and suspensions contained 38.8 ± 4.0% PMNs, 57.2 ± 4.1% lymphocytes, 2.4 ± 0.9% monocytes, and 1.8 ± 0.6% eosinophils. Cells were then resuspended in 2 ml 0.5% HTAB, sonified, freeze–thawed, and kept frozen at −80° until assayed for MPO.

In order to obtain extracts of MPO from the neutrophils in sponges, neutrophils were pooled from four to five sponges at various times. The cells were counted electronically and differential count was done. The suspension was then centrifuged at 500*g* for 5 min and the supernatant removed. The cell pellet was suspended in 2 ml 0.5% HTAB, sonified, freeze–thawed, and frozen until MPO assay.

MPO assay. Specimens were centrifuged at 40,000*g* for 15 min, and 0.1 ml of the supernate was mixed with 2.9 ml of 50 m*M* phosphate buffer, pH 6.0, containing 0.15 mg/ml *o*-dianisidine dihydrochloride (Sigma Chemical Co., St. Louis, Mo.) and 0.0005% hydrogen peroxide (Mallinckrodt, Paris, Ky.). The change in absorbance at 460 nm was measured with a Beckman DU spectrophotometer (Beckman Instruments Lab., Fullerton, Calif.). One unit of MPO activity was defined as that degrading 1 μmole of peroxide/min at 25° (12).

Statistical analysis. Data were analyzed by means of Student's *t* test.

Results. The inflammatory response induced by an implanted polyvinyl sponge includes the increased outflow of stored neutrophils from the bone marrow into the blood and the migration of those neutrophils to the sponge and the adjacent tissue. Furthermore, neutrophils may migrate to the incision made to insert the sponge. In order to assess the magnitude and kinetics of these events, we evaluated the changes in blood neutrophil content and NSP size, as well as neutrophil accumulation within the sponge and its vicinity, for a 24-hr period following sponge implantation.

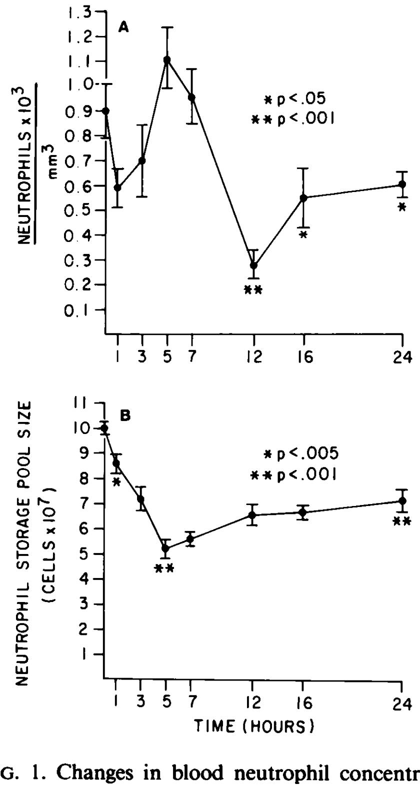

FIG. 1. Changes in blood neutrophil concentration (A) and total marrow neutrophil storage pool size (B) after sponge implantation. Each point represents the mean value for groups of 6–25 mice. The brackets indicate the SEM. The values for the neutrophil storage pool after 3, 7, 12, and 16 hr also differed from controls at the $P < 0.001$ level.

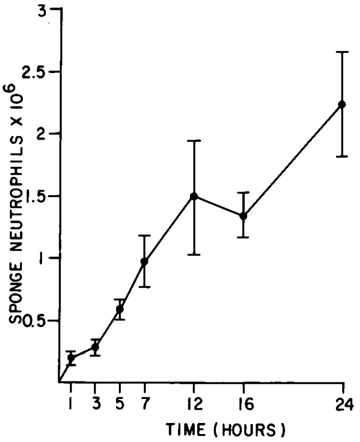

FIG. 2. Accumulation of intact neutrophils in implanted sponges. The maximum sponge content of neutrophils was observed after 24 hr ($P < 0.05$). Each point represents the mean value for groups of 6–14 sponges. The brackets represent the SEM.

The effect of sponge implantation upon blood neutrophil concentration and neutrophil storage pool (NSP) size. Variations in caval blood neutrophil concentration occurred promptly after sponge implantation (Fig. 1A). The mean neutrophil concentration fell 1 hr after sponge implantation ($P < 0.1$) with six of eight values occurring below the control range. After 5 hrs, the mean neutrophil concentration was higher than that of controls. Maximum neutropenia to 31% of control, was observed 12 hr after sponge implantation ($P < 0.001$), and neutropenia persisted thereafter ($P < 0.05$).

Sponge implantation induced a reduction in the marrow's content of mature neutrophils, and all groups of mice bearing sponges exhibited a significant reduction in NSP size from 1 to 24 hr after implantation (Fig. 1B). The NSP size was reduced by 48% of the mean control value within 5 hr, when $4.81 \pm 0.35 \times 10^7$ ($\bar{x} \pm$ SEM) neutrophils were missing from the NSP.

Accumulation of intact neutrophils in the sponge fluid. The mean number of intact neutrophils accumulating in the sponges increased throughout the study period (Fig. 2), with maximum values occurring after 24 hr ($P < 0.05$). The values at any given time dif-

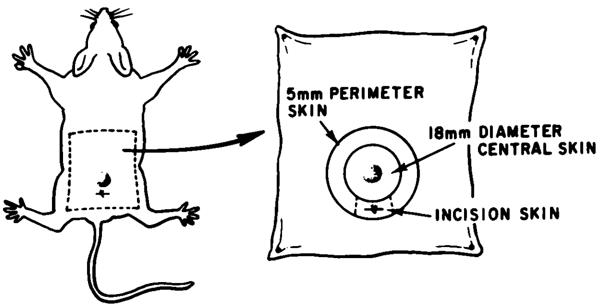

FIG. 3. Assessing the combined inflammatory lesion after sponge implantation. Both sponge and various skin specimens (central skin, incision skin, and perimeter skin) were individually assayed for MPO.

fered considerably with 6- to 12-fold differences between the lowest and the highest values. Interestingly, the mean value which was highest (2.21 ± 0.41 × 10^6 neutrophils per sponge after 24 hr) only accounted for 5% of the number of cells missing from the NSP at its maximum depletion.

Neutrophil accumulation in the combined inflammatory lesion (sponge + skin). Because the degree of depletion of the NSP greatly exceeded the number of neutrophils in the sponge fluid, we postulated that tissues adjacent to the sponge might be the site of significant neutrophil migration. In order to test this hypothesis, we utilized an assay for neutrophils based upon myeloperoxidase (MPO), an enzyme marker for neutrophils. With this method, the tissue and sponge could both be evaluated for the accumulation of neutrophils. First, the MPO content of a 14-mm-diameter circle of skin overlying the sponge was determined. The maximal accumulation of MPO in tissues occurred 7 hr after sponge implantation (1.39 ± 0.16 units MPO), with no significant change thereafter. Thus, the maximal accumulation of neutrophils in tissues occurred after 7 hr, whereas the accumulation of neutrophils in sponge fluid continued to increase throughout the 24-hr period.

In order to determine the geographic extent of the neutrophil response and the relative proportion of neutrophils in the sponge or tissue, further studies were carried out 3, 7 and 24 hr after sponge placement. Skin in the sponge vicinity was obtained in a circumferential fashion: a central 18-mm-diameter circular area overlying the sponge, the 5-mm perimeter to it, and the incision area (Fig. 3). The skin specimens were then assayed for MPO. The 5-mm outer skin perimeter from 15 animals with sponges had the same content of MPO as skin from control animals (0.04 ± 0.01 units MPO), indicating that the neutrophil inflammatory response did not extend beyond the incision and 18-mm central area. The pattern of MPO accumulation for the incision and overlying skin is shown in Fig. 4, where the MPO of sponges is also shown. It can be seen that neutrophils migrated to each of these sites. In order to determine the relationship of the neutrophil content of the sponge to that of the combined lesion (sponge + skin) the MPO of sponges was compared with the sum of the MPO content of the central skin, the incision skin, and

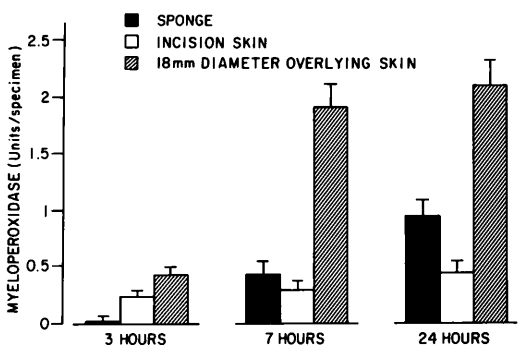

FIG. 4. The MPO content of implanted sponges and surrounding skin. Each bar represents the mean value for 5–10 experiments. The brackets display the SEM. The MPO content of equivalent skin samples from unmanipulated controls was incision area, 0.03 ± 0.01 units MPO/specimen; 18-mm overlying skin, 0.11 ± 0.03 units MPO/specimen.

the sponge. The MPO of sponges was never more than 34% of the MPO in the sponge and skin combined. Furthermore, the ratio of MPO from sponges to the MPO of combined lesions varied from 0.3 to 13% after 3 hr; from 5 to 22% after 7 hr; and from 24 to 34% after 24 hr. Thus, the migration of neutrophils to the sponge itself represents an incomplete and variable estimate of the response to the entire lesion.

Estimation of the number of neutrophils in lesions. The extraction of MPO from lesions reflects the net accumulation of this enzyme as it is contributed by migrating neutrophils. Monocytes also contain MPO, but their contribution to the MPO of lesions is minimal since we have previously shown that rodent monocytes contain 1.9% of the MPO in rodent neutrophils (8) and furthermore, monocytes only comprised 1.6 ± 0.2% of the cells in the lesions.

Therefore, an estimate of the neutrophil content of the combined lesion (sponge + skin) can be obtained by dividing the value for MPO extracted from the lesion by the MPO of individual neutrophils. Two approaches to these calculations are described below.

An estimate of the minimal number of neutrophils which must have migrated to a lesion to contribute the MPO there can be determined from the MPO content of blood neutrophils and the MPO content of the lesions.

In five experiments, the MPO content of blood neutrophils was $2.50 \pm 0.34 \times 10^{-7}$ units per cell. By dividing blood neutrophil MPO content into the mean value for the total extractable MPO of the lesions at the time of maximal sponge neutrophil content (24 hr), it was estimated that a minimum of 1.39×10^{7} neutrophils had migrated to the lesions. In this way, at least 29% of the neutrophils missing from the NSP may be accounted for in the combined lesions (8% in the sponge alone).

In order to test the hypothesis that neutrophils lost MPO after migrating to lesions, the MPO content of intact neutrophils recovered from implanted sponges was determined. In six experiments, neutrophils collected from sponges implanted for 7 and 24 hr exhibited similar MPO values: $0.93 \pm 0.06 \times 10^{-7}$ and $1.07 \pm 0.07 \times 10^{-7}$ units per neutrophil, respectively ($P < 0.3$). A value of $1.00 \pm 0.05 \times 10^{-7}$ units per neutrophil was obtained by combining these data and thus it was found that neutrophils from lesions had lost 60% of their MPO. Other studies suggest that *little of the lost MPO survives* extracellularly in active form (13). If this reduced MPO value for neutrophils is divided into the mean value for MPO extracted from combined lesions it can be estimated that at least 3.48×10^{7} neutrophils had accumulated at 24 hr. In this way 73% of the neutrophils missing from the marrow can be accounted for in the combined lesions (*20% in the sponge*).

Discussion. A number of approaches have been developed to investigate the migration of neutrophils during the acute inflammatory process *in vivo.* A useful model was introduced by Coste *et al.* in 1955 in which polyvinyl sponges were implanted subcutaneously in animals and the sponges acted as attractants for neutrophils (4). Thus, at the end of an observation period the implanted sponges could be recovered and the neutrophils in the sponge exudate quantified. Implementation of this method has enabled investigators to evaluate the effect of complement depletion (14), a human plasma fraction (15), and various drugs upon neutrophil migration *in vivo* (4, 6, 7). In addition, the *in vivo* survival and migration of transfused neutrophils have been studied (16, 17). The sponge method has also been used to define a defect of neutrophil migration in neonatal rats (18), and bacteria have been added to sponges to examine bacterially induced neutrophil migration (19, 20). These studies have concentrated upon changes in the blood, and the accumulation of neutrophils in the sponges themselves. We postulated that sponge implantation might stimulate neutrophil release from the marrow, and that some neutrophils might not migrate to the sponge itself, but remain in the tissue surrounding it. Thus, we used a variety of quantitative measurements to define each of these events.

Sponge implantation did induce the release of marrow neutrophils; a 48% reduction in the NSP was noted within 5 hr of sponge implantation. Thereafter, the NSP increased, consistent with the hypothesis that the release stimulus was greatest during the initial 5–7 hr after sponge implantation. Although the NSP decreased, the neutrophil concentration within the circulating blood did not significantly increase and in fact decreased. This observation does not exclude the possibility of an increase in the total blood neutrophil mass, since many neutrophils may have been released, entered the blood, and then were included in an enlarged marginal neutrophil pool (21, 22).

In order to evaluate the migration of neutrophils to the sponge and perisponge tissue, we employed two methods: (a) measurement of the number of intact neutrophils within the sponge and (2) estimation of the neutrophil content of the sponge and tissues by assaying for the neutrophil marker enzyme myeloperoxidase (MPO). The accumulation of MPO in the perisponge tissue reached maximum levels within 7 hr of sponge implantation, occurring during the period of maximum release of neutrophils from the bone marrow. The accumulation of neutrophils within the sponge itself displayed a different kinetic pattern, with maximum values 24 hr after implantation. This difference in the time of maximal accumulation of neutrophils in the tissue and the sponge is compatible with the hypothesis that the tissues are the initial site of major neutrophil migration, and neutrophils may then migrate from the tissue to the sponge, rather than by direct cell movement from perisponge vessels. Assays of MPO deposited in tissue by migrating neutrophils revealed that neutrophils accumulated in the sponge, the skin overlying the sponge, and the incision site. The MPO content of tissues and sponges were compared, and it was found that the sponges only contained 0.3–33% of the MPO of the combined lesions (sponge + skin). Whether similar results might be obtained in studies with multiple sponges in other animals (7, 16, 17) remains to be tested.

The measurement of the marrow's content of neutrophils provides a means of estimating the release of neutrophils after sponge implantation. At least 4.8×10^7 mature neutrophils left the marrow in response to the sponge stimulus. However, this represents a minimal estimate since neutrophil proliferation may mask some of the neutrophil release by replacement of the existing NSP with new cells. Of additional interest is the proportion of released neutrophils which reach the sponge or combined lesion (sponge and skin). Such an estimate can be made from the MPO found at these sites. Since the induced lesions were almost exclusively composed of neutrophils, their MPO contents can be taken as indices of neutrophil accumulation. In order to utilize the MPO value of the sponge and skin to estimate neutrophil accumulation, it was necessary to determine the average MPO per neutrophil. We postulated that the MPO for circulating neutrophils was different from that for neutrophils which had accumulated in the tissue and the sponge, and indeed neutrophils within the sponge had lost 60% of their MPO activity. Therefore, we calculated two rough estimates for neutrophil accumulation: one based upon the MPO of neutrophils still in the blood, and the other based upon the residual MPO of neutrophils in the sponge. In this way, we estimate that from 8 to 20% of released neutrophils could be accounted for in the sponge at 24 hr, whereas 29–73% were contained within the combined lesion. However, these calculations may provide an underestimate of actual neutrophil migration since they did not include measurement of at least two factors: (a) MPO which may have been degraded, and (b) the neutrophil content of tissue underneath the sponge.

From the present study, it is concluded that implantation of a standardized sponge stimulus in mice induces significant marrow neutrophil release, with subsequent accumulation of neutrophils both in the sponge and the surrounding tissue as well. The sponge itself attracts a small and variable proportion of the neutrophils migrating to the entire lesion. These studies provide an initial view of the acute inflammatory process to foreign body (sponge) implantation by defining the various components of the neutrophil response. The present approach may be useful in studying the factors which regulate neutrophil release from the marrow, and also provides a means for studying the tissue phase of neutrophil migration quantitatively.

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