Lymphocyte Participation in Wound Healing

Morphologic Assessment Using Monoclonal Antibodies

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To investigate lymphocyte participation in wound healing, the migration of T lymphocyte subsets into healing wounds and subcutaneously implanted polyvinyl alcohol sponges was studied. Frozen sections of 5-, 7-, and 10-day-old incisional wounds and sponges from Lewis rats were stained with mouse anti-rat monoclonal antibodies. Cellular staining to OX1 (all leucocyte), W3/25 (helper/effector T lymphocytes), and OX8 (suppressor/cytotoxic T lymphocytes) was quantitated in two arbitrarily defined areas based on maximal cellular infiltration: (1) the superficial wound, down to and including the papillary dermis, and (2) the deep wound, the reticular dermis. Five-day wounds were significantly more cellular than 10-day wounds in the deep portion ($p < 0.05$) and somewhat more cellular in the superficial section ($p < 0.10$). Approximately 2:1 W3/25 to OX8 ratios were noted for wound strips on all days. At 5 and 10 days there are twice as many W3/25 and OX8 labeled cells in the deep wound as in the superficial portion. At 7 days there is a peak in surface W3/25 and OX8 lymphocytes, whereas the deep population remains constant. Seven- and 10-day sponge granulomas demonstrate ratios similar to the wound strips (5-day sponge lymphocytic infiltration was insufficient to count). The data demonstrate that lymphocyte subpopulation participation in wound healing is a dynamic and distinctive process.

THE NORMAL HEALING OF WOUNDS involves a cascade of cellular events. Although lymphocytes predictably migrate into wounds,[1] their role in the healing process is poorly defined. Antigen-stimulated lymphocytes produce lymphokines, which have been shown to influence *in vitro* fibroblast replication and collagenous protein synthesis.[2,3] Evidence also exists that alterations in host immune response can alter the course of fibroplasia after wounding.[4,5] However, direct *in vivo* proof of lymphocyte participation in wound healing is lacking.[6]

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The purpose of this experiment was to elucidate the role of lymphocytes in wound healing by characterizing morphologically the T lymphocyte subpopulations that migrate into wounds at various stages of healing. The aims of this study were: (1) to determine the ratio of T helper/effector:T suppressor/cytotoxic lymphocytes present in wounds, and (2) to study the dynamics of T lymphocyte subsets during wound healing. This was accomplished by using specific monoclonal antibodies to examine T lymphocyte subsets in a rat wound healing model.

Materials and Methods

Male Lewis rats (Harlan Sprague-Dawley, Walkersville, MD), weighing 250–300 g were individually housed at constant temperature (25 ± 1 C) and humidity (40–50%). All rats were fed a complete laboratory chow, Teklad 4% Mouse-Rat Diet (Teklad Test Diets, Madison, WI) and tap water *ad libitum.* The rats were allowed at least 1 week of acclimatization to our laboratory conditions before being used in the experiments.

Under pentobarbital anesthesia (4 mg/kg body weight i.p.) the backs of the rats were shaved and then scrubbed with povidone iodine solution. Using clean conditions, 7-cm dorsal midline wounds were made sharply down to the level of the fascia. At the upper poles of the wound, two sterile, moistened polyvinyl alcohol sponges were placed into subcutaneous pockets. The wounds were then closed with seven interrupted 4-0 stainless steel sutures.

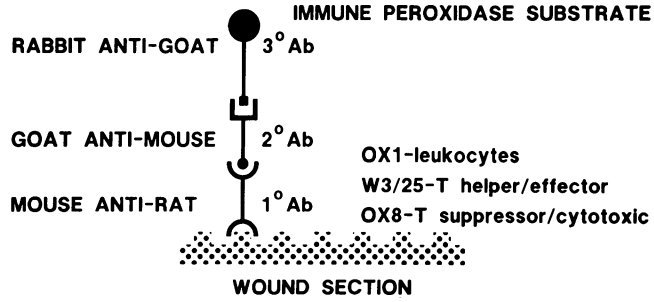


FIG. 1. Diagram of the immunohistochemical staining method used for the identification of antigens on the surface of wound cells by the use of monoclonal antibodies. Three reagents are used: (1) primary antibody, (2) second antibody capable of binding to the first, and (3) biotinylated third antibody. This can then form a biotin–avidin peroxidase complex.

The rats were killed at 5, 7, or 10 days after wounding. A segment of the healing wound was excised and frozen in isopentane cooled to −150 C with liquid nitrogen. Sponge granulomas were cleared of surrounding connective tissue and similarly frozen. Wound blocks and sponges were cut in a cryostat at −20 C. Serial sections were stained using an Avitin–Biotin complex.[7] Briefly, sections were incubated with mouse anti-rat primary monoclonal antibodies (Pel Freez, Rogers, AR) at a 1:50 dilution. The following monoclonal antibodies were used: OX1 against the rat leukocyte common antigen expressed by thymocytes, T and B lymphocytes, and macrophages; W3/25 against the helper/effector T lymphocyte subset; and OX8 against suppressor/cytotoxic T lymphocytes.[8] Sections were then treated with a 1:200 dilution of goat anti-mouse IgG antibody (Calbiochem, San Diego, CA), a 1:800 dilution of tertiary biotinylated rabbit anti-goat IgG antibody (Vector Laboratories, Burlingame, CA), and the Avitin-Biotin immune peroxidase complex (Vector Laboratories, Burlingame, CA).

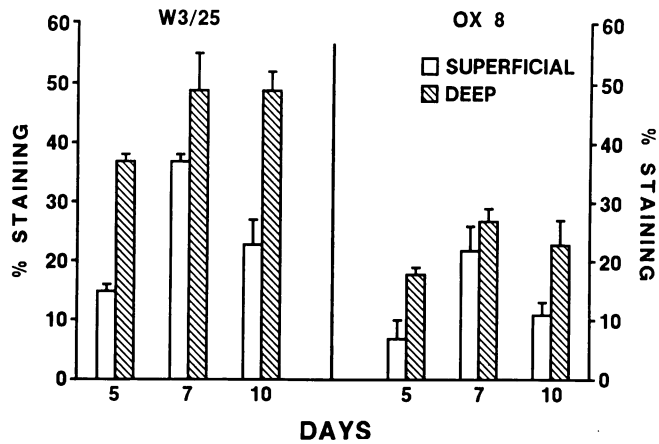


FIG. 2. Mean percentage of mononuclear wound cells stained with W3/25 or OX8 in the superficial and deep portions of the wound.

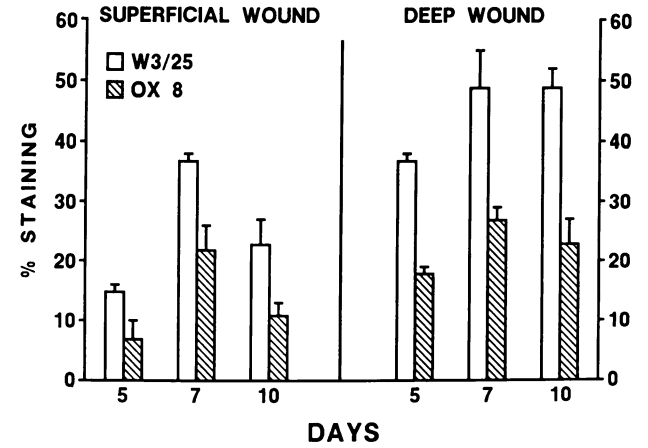


FIG. 3. Mean percentage of mononuclear wound cells stained with W3/25 or OX8 comparing the superficial and deep aspects of the wound.

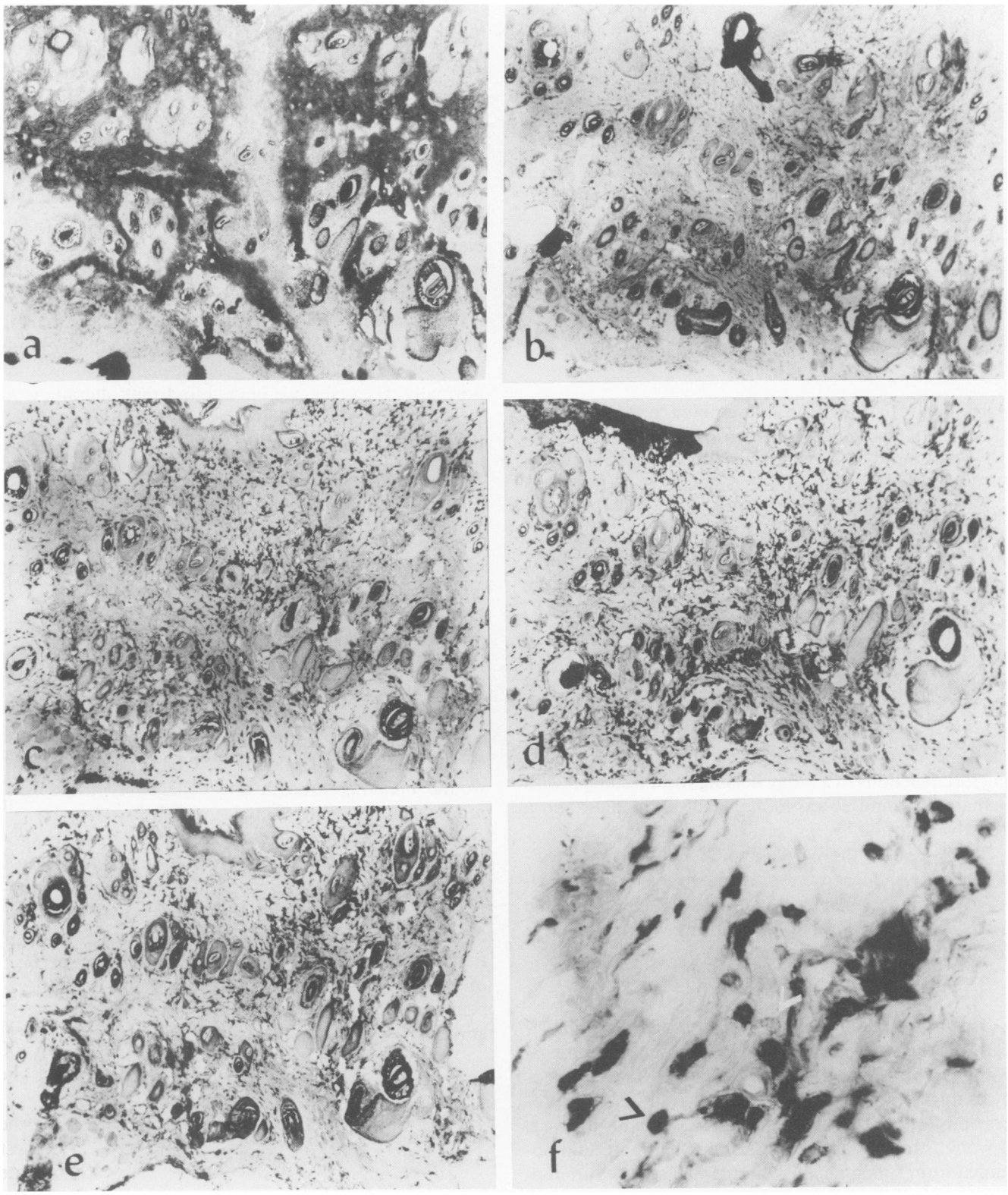
All incubations were for 30 minutes at room temperature in a humidified atmosphere. Figure 1 is a schematic representation of the staining technique used. Control sections received the same treatment except for the primary monoclonal antibody.

Sections stained with hematoxylin and eosin were studied to identify the wound or the location of cells in the sponge matrix. The mononuclear cell infiltrate of 5-, 7-, and 10-day-old wounds was ranked blindly using the OX1 stains. Cells stained with monoclonal antibody were counted in two areas of the wound: the superficial wound, down to and including the papillary dermis, and the deep wound, the reticular dermis. This division was made arbitrarily as it best reflected the areas of highest cellular infiltration. Cellular staining was quantitated in a blind fashion as follows: the section stained with OX1 that marks all leukocytes was counted. This allowed localization of the mononuclear wound cell populations to be counted in the subsequent serial sections (stained with the W3/25 and OX8 monoclonal antibodies). The exact number of cells counted was dependent on the overall mononuclear cell infiltrate of the wound or sponge. Between 100 and 300 round cells were counted in each area of the wound and the percentage of positivity by stained cells was determined. Likewise, 100–300 round cells were counted in each sponge section and the percentage of positive cells was determined.

Each data point reported in the study represents the mean of six separate wounds. The Mann-Whitney test was used to rank blindly the mononuclear cell infiltrate using the OX1 antibody.

Results

Five-day wounds had a significantly heavier mononuclear cell infiltration than 10-day wounds in the deep



FIGS. 4A–F. Composite of serial sections of a segment of a 7-day healing wound. *B–F.* Nuclei were counterstained with polychrome methylene blue. *A.* Hematoxylin-eosin stain of the healing wound. *B.* Negative control. Section stained using the biotin–avidin peroxidase technique excluding the primary monoclonal antibody. *C.* OX1. Section stained with primary monoclonal antibody against all leukocytes. Stained cells can be seen in all regions of the wound. *D.* W3/25. Staining is more marked in the deep rather than the superficial aspect of the wound. *E.* OX8. Fewer cells are stained compared with *D. F.* High power of W3/25. Note the discrete membrane staining (black arrow) *versus* unstained cells (white arrow).

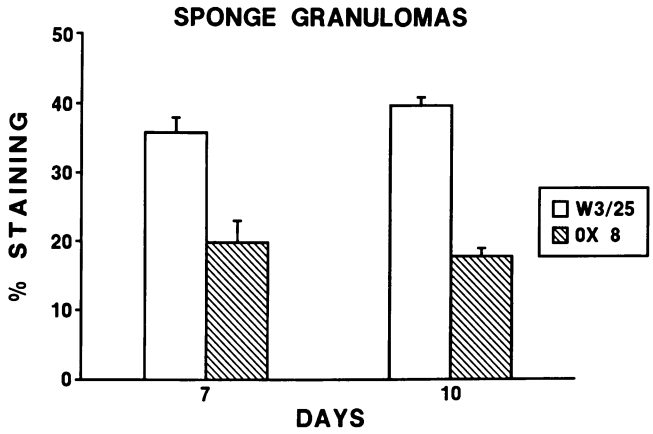


FIG. 5. Mean percentage of mononuclear wound cells stained with W3/25 and OX8 in the sponge matrix at 7 and 10 days.

portion ($p < 0.05$). This was also noted in the superficial section, although not as severe ($p < 0.10$). For 5-, 7-, and 10-day wounds (Fig. 2), lymphocytes labeled with W3/25 predominated over cells bearing the OX8 marker in approximately 2:1 ratios of the mean percentage positive cells in the superficial portions of the wound. There were twice as many W3/25 and OX8 labeled cells in the deep aspect of the wound as in the superficial portion at day 5 (W3/25, 37:15; OX8, 18:7) and day 10 (W3/25, 49:23; OX8 23:11). However, at day 7 there was a peak in W3/25 and OX8 labeled T lymphocytes in the superficial area, whereas the lymphocyte population in the reticular dermis (the deep portion) remained constant (Fig. 3). Figure 4 is an example of a 7-day wound. This is a composite picture showing serial sections of a 7-day healing wound stained with hematoxylin and eosin, monoclonal antibodies, and a negative control.

T cells bearing the W3/25 and OX8 markers in the 7- and 10-day sponges were present at the same approximate 2:1 ratio as seen in the wound strips (Fig. 5). In the 5-day sponge, the lymphocytic infiltrate was insufficient to count.

Discussion

This experiment was designed to assess morphologically the migration of T lymphocyte subsets into surgical wounds. We also hoped to demonstrate that lymphocytes migrate to the implanted sponges in the same proportions as they do into the wounds. Were this the case, it would support the hypothesis that cells and fluid obtained from subcutaneously implanted sponges are representative of the cellular infiltrate into the wound itself.

T lymphocyte subsets positive for the W3/25 (helper/effector) and OX8 (suppressor/cytotoxic) markers were found to migrate to the superficial and deep portions of the wound in a 2:1 ratio. These ratios are similar to those reported in rat lymph nodes (1.7–2.3:1),[8,9] but lower than the ratio found in rat peripheral blood lymphocytes (4.2:1).[10] Wound lymphocyte migration is a dynamic process as populations of both W3/25- and OX8-positive T lymphocytes peak in the superficial area of the 7-day-old wound. This is similar to previous data that examined the dynamics of wound lymphocyte infiltration[1]; that study, however, did not study T lymphocyte subpopulations. Lymphocyte subsets infiltrate the implanted sponges in the same 2:1 ratio as seen in the wound, suggesting that the sponge granuloma cellular infiltrate reflects the cellular dynamics of the wound.

The role of lymphocyte participation in the healing process is not clear. Earlier work[6] suggested that wounds of rats depleted of lymphocytes healed dorsal wounds normally. However, we have found that alterations in the host's systemic immune responses influence wound healing. Treatment of rats with immunosuppressive doses of cyclosporin A (which has been shown to decrease the normal T helper/T suppressor ratios)[11] impairs wound healing.[5] Further, adult thymectomy, which abrogates the induction of suppressor T cells, enhances wound healing.[4] *In vitro* evidence suggests that lymphocytes can regulate fibroblast replication and collagen protein synthesis.[2,12] Both stimulated and unstimulated lymphocytes secrete soluble factors, lymphokines, that are reported to either inhibit or enhance fibroplasia.[12] However, the role these factors may play in *in vivo* wound healing remains to be elucidated.

More recently, work from our laboratory has demonstrated that *in vivo* depletion of T lymphocytes impairs wound breaking strength and collagen synthesis in a mouse wound healing model (unpublished data).

The current experiments demonstrate that lymphocytic infiltration of healing wounds is a dynamic process that peaks at 7 days after wounding. Although the morphologic methodology used herein does not allow for direct evaluation of lymphocyte-fibroblast interaction, it may be useful in studying whether the detrimental effect of lympholytic agents such as steroids or chemotherapeutic agents on wound healing is mediated by alterations in wound lymphocyte migration.

Addendum

Since carrying out these studies, a paper has been published using a similar technique to study the appearance of W3/13-positive lymphocytes in healing femoral fractures in rats.[13]

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