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A Novel, Vasoactive Product and Plasminogen Activator from Afferent Lymph Cells Draining Chronic Inflammatory Lesions[1] (40495)

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Chronic inflammatory sites are characterized by the accumulation of blood-derived mononuclear cells. However, such sites are much more dynamic than the histopathology suggests. Smith *et al.* (1) showed that these areas in sheep were characterized by a greatly increased migration of cells through the lesion. They were able to continuously collect afferent lymph from such a subcutaneous focus for periods of up to 2 months. During this time, the cell output in the lymph increased approximately 25-fold. At the maximum, this traffic equalled the migration through a normal lymph node and the accumulated mass of cells which entered the lymph was many times the mass of the lesion itself. The cells which comprise the normal afferent lymph compartment include 5–20% mononuclear phagocytic cells and the rest lymphocytes, as described by Hall and Morris (2) and this ratio does not change appreciably during the development of these lesions. The population of mononuclear phagocytic cells does not readily pass through lymph nodes and is therefore virtually absent from efferent lymph, even though the regional node may be stimulated by the inflammatory focus and may contain basophilic lymphoblast cells. The increase in regional blood flow (hyperemia) to the area of antigenic challenge has been shown to correlate well with concomitant increases in lymphocyte traffic through the region (2). This relationship between hyperemia and lymphocyte recruitment has been shown to hold both in lymphoid and nonlymphoid tissues (3). The enhancement of blood flow will cause diversion of greater numbers of lymphocytes through the microvasculature of the region and could allow for more lymphocytes to enter the tissue space without altering the efficiency of the extraction. Since the blood flow has been shown to increase in response to the presence of antigen, this would indicate that the vasoactive mediator of this hyperemia would be the product of a cell involved in the immune response. We report here the finding of such a vasoactive product which appears to be distinct from the E-type prostaglandins and from plasminogen activator. Using a microsphere skin test assay (4, 5) to quantitate vasoactivity, cell products from afferent and efferent lymph cells migrating from chronic inflammatory sites were compared and it was found that only afferent cells produced vasoactive material. Secondly, afferent lymph cells produced a plasminogen activator while efferent cells did not.

Materials and methods. Four sheep were injected with Freund's complete adjuvant containing 0.5 human doses of bacillus Calmette Guérin (BCG) per ml. A total of 1 ml was injected in two subcutaneous sites in the hock of each hind leg. An afferent lymphatic vessel (identified by injecting 0.2 ml of 2% Evans Blue in 0.9% NaCl directly into the granuloma) was cannulated (2), 13, 18, 45 and 65 days after the inflammation was initiated. These preparations flowed for varying periods ranging from hours to several weeks. If stoppage occurred, the cannulae were repositioned. Lymph samples were not utilized until 1 day or more after anaesthetic. Only mononuclear cells and in some cases occasional red cells were present in these preparations. The hourly cell output was 10–30 million and flow rates of up to 5 ml/hr were recorded. It was clear that a portion of the inflammatory site was being drained, since such a cell output is approximately 10–30 times normal. Cells from afferent or efferent (2) lymph were washed twice with phosphate-buffered saline (PBS) and resuspended in Medium 199 with Earle's salts, glutamine and antibiotics at a concentration of 1×10^7 cells per ml. Cells were cultured for 24 hr at 37° in a humidified incubator with 5% CO_2 and

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95% air. The culture supernatants were injected into the dorsal skin of rabbits at various times prior to administration of microspheres, and vasoactivity assayed as previously described (5). Briefly, rabbits were anaesthetized with ethyl carbamate, a polyethylene catheter (PE 50, Clay Adams, Parsipanny, NJ) was advanced via the right common carotid artery into the aortic arch, and 10 μm-sized, Sn^{113}-labelled, radioactive microspheres introduced into the arterial circulation. Animals were sacrificed with an overdose of sodium pentobarbital, the skins removed from the back, and the test and normal sites punched out with a cork borer. The radioactivity localized in these skin sites was measured on a gamma scaler (Intertechnique, Cg30). The specific activity of the microspheres was determined. Blood flow is expressed as a ratio of the number of microspheres impacted in injected sites to the number of microspheres impacted in normal sites.

Conditioned media from cells of afferent and efferent lymph were assayed for fibrinolytic activity. 1×10^7 afferent or efferent cells were cultured in ^{125}I-fibrin-coated multi-well Linbro plates in 2 ml of medium. Fibrinolysis was quantitated by withdrawing 100 μl aliquots of medium at various times. The amount of ^{125}I-fibrin solubilized is expressed as the percentage of the total radioactivity that can be released by an excess of trypsin.

These same supernatants were incubated with 10^{-3} *M* diisopropylfluorophosphate (DFP) for $\frac{1}{2}$ hr at 37°, dialyzed against PBS and subsequently assayed for vasoactivity and fibrinolytic activity.

Cells from afferent lymph were cultured for 24 hrs in the presence of 6×10^{-7} *M* indomethacin, the supernatant was dialyzed against PBS and assayed for vasoactivity and fibrinolytic activity.

Results. Supernatants of cultures of washed, stimulated, afferent lymph cells were assayed for vasoactivity. When the supernatants were injected intradermally into the dorsal skin of rabbits, the kinetics of the resulting hyperemia were markedly different from those displayed by prostaglandin E_1 (PGE_1) (Fig. 1). Whereas PGE_1 showed a peak activity at 45–60 min after injection (6), and had subsided by 90 min, the hyperemia produced by the supernatants of stimulated

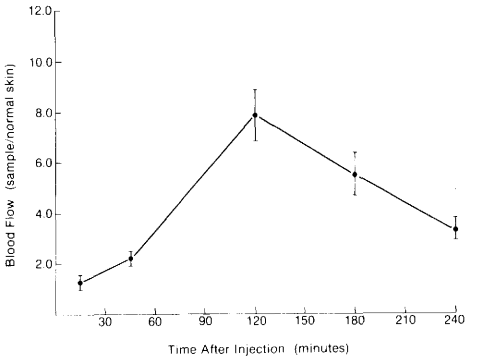

FIG. 1. The circles represent mean ± SEM values (n = 6) for afferent cell culture supernatants.

afferent cells showed a slower onset and reached peak activity near 120 min. On the other hand, supernatants of cultures of stimulated efferent lymph cells (100% lymphocytes) did not cause hyperemia when injected.

Using the same microsphere assay, the potency and duration of the vasoactive effects of several well characterized molecules such as histamine, bradykinin, epinephrine, and E and F prostaglandins have been studied, including the effects of trauma or saline injections (5–7). The lymph cell product has characteristics which are different from all of these. A similar unknown hyperemia-inducing material was first observed *in vivo* in lymph plasma draining tuberculin reactions (Vadas, unpublished observations). When lymph plasma draining sites of chronic inflammation was tested directly, it exhibited vasoactivity, whereas normal lymph plasma did not alter the blood flow to rabbit skin sites (Vadas, unpublished observations).

Stimulated afferent lymph cells tested on ^{125}I-labeled fibrin plates for fibrinolytic activity were found to produce a plasminogen-dependent and time-dependent lysis (Fig. 2). This lysis was also dependent on the cell concentration. The active fibrinolytic material is plasminogen dependent and is probably similar to the plasminogen activator produced by macrophages from BCG-primed mouse peritoneal exudates (8). This enzymatic activity was not found in stimulated efferent cell culture supernatants. Enzyme activity was not found in lymph plasma draining a granuloma, presumably due to inhibition by plasma inhibitors. Further data

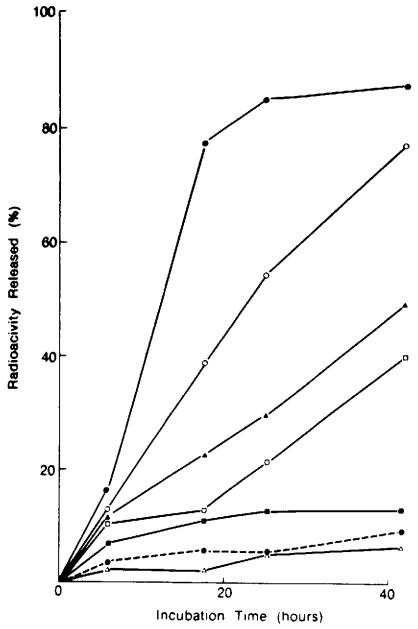

FIG. 2. Time course of fibrinolysis and dependence of plasminogen activator activity upon plasminogen concentration. The plasminogen concentrations were ●——● 50 μg, ○——○ 25 μg, ▲——▲ 10 μg, □——□ 5 μg, ■——■ no plasminogen; ●-----● efferent cells + 50 μg plasminogen; △——△ medium alone. All incubations were performed in duplicate and the results were averaged.

on the plasminogen activator will be reported separately (Wasi, to be published).

The plasminogen activator was inhibited by 10^{-3} *M* DFP treatment, but the vasoactive material retained all of its activity. Furthermore, the vasoactive agent did not depend upon the presence of plasminogen or fibrinogen. Neither the vasoactive material nor the enzyme component is suppressed by culturing the cells with indomethacin at a concentration shown by others to partially inhibit prostaglandin synthesis (10).

Discussion. It has been experimentally and quantitatively shown that the blood flow to cellular hypersensitivity sites in sheep, rabbits and guinea pigs increases several fold as the lesion develops (3, 5). Dermal lesions produced in rabbits by the injection of 0.1 ml of Freund's complete adjuvant show enhancement of blood flow 8 times that of equivalent sized areas of normal skin and this increased flow persists for at least 2 weeks (5). The vasoactive material described here may contribute to these blood flow changes. Since the conditioned medium of afferent but not efferent lymph cells contains the hyperemia-inducing activity, and afferent lymph cells differ from the efferent population by the presence of approximately 15–20% macrophages in the former, this would tend to implicate the macrophages in the elaboration of this activity. Moreover, based on the similarity of onset and duration of the hyperemia produced by either the *in vitro* or *in vivo* derived materials, we suspect that the material which appeared in the lymph plasma was also macrophage derived. The inactivation of plasminogen activator, but not the vasoactive material by DFP treatment demonstrates that intact serine esterase activity is not an essential requirement for expression of vasoactivity. The inability of indomethacin to inhibit production by afferent cells of the vasoactive material would indicate that this material is not a prostaglandin, either free or bound. It is, however, possible that the vasoactive component stimulates prostaglandin production in the skin of the test rabbit (11) and this is being investigated. Although there are some similarities between the vasoactive agent and the lymphokine skin reactive factor, these data favour the interpretation that macrophages are the cellular source and not lymphocytes. Polverini *et al.* (9) have demonstrated the presence of a macrophage secretory product which acts on the microvasculature. This mediator induces neovascularization of guinea pig corneas. This work establishes a precedent for the ability of an activated macrophage product to alter microvasculature at the site of chronic inflammation.

Summary. A vasoactive product of a cell type present in afferent and not efferent lymph has been described. The kinetics of the resultant hyperemia following intradermal injection of the material distinguish this activity from histamine, bradykinin or prostaglandin E_1. Furthermore, the activity appears to be distinct from plasminogen activator and prostaglandins on the basis of studies with DFP and indomethacin. This vasoactive product may mediate blood flow alterations following antigenic challenge. A relationship between antigen-induced increased blood flow and increased lymphocyte traffic has

been described for both fixed lymphoid tissue and induced cellular hypersensitivity sites. It is our contention that the regulation of the blood flow to such regions may be of fundamental importance in governing the number of inflammatory cells in an area and the rate of extravasation of blood plasma.

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