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17. Five unimmunized rabbits were anesthetized and a midline abdominal incision performed to expose the bowel. A 30-ml subcutaneous injection of isotonic saline was given to replace fluid loss during the procedure. After an intraluminal injection of 20 ml of phosphate-buffered saline (PBS) to clear chyme distally, two contiguous 15-cm segments of small intestine were surgically isolated. The mesenteric veins that drained each section were identified, and any collateral vessels not draining into the vessel to be cannulated were ligated. An intravenous injection of 500 units of sodium heparin per kilogram of body weight was given before the first cannulation to allow collection of blood without clotting. The major mesenteric vein draining the isolated segment of intestine was then cannulated, and a 1-min "prebleed" was collected. Within the first minute of collection, the loop section was injected with 1.0 ml of either control or immune intestinal secretions (a quantity representing less than one day's production of immunoglobulin), followed by the injection of an average of 1.5×10^6 cpm of the ^{125}I-pHP-AAF (approximately 0.33 ng) at the start of the second minute. The venous drainage was collected in 1-min fractions into heparinized 1.5-ml centrifuge tubes for the next 30 min, after which the section of bowel was removed, and the residual fluid contents of the section were collected. The bowel section was placed in 10 ml of PBS until processing of the second loop was complete. The second loop section was treated identically to the first section, with only the source of intestinal secretion differing. Injection of immune and nonimmune fluid was alternated distally and proximally, as well as temporally. The distribution of radioactivity (Table 1) in the blood and tissue samples was estimated as follows. (i) The blood samples were centrifuged at 10,000*g* for 30 s and subjected to gamma counting. (ii) The wet weight of each loop section was determined, and a small portion was removed and weighed before gamma counting. (iii) The total and antibody-bound counts in 100 μl of the fluid remaining in the loop section were approximated by separating the free tracer from macromolecular bound tracer with cold dextran-coated charcoal as described (*10*). Comparative recoveries of radiotracer averaged less than a 3% difference between loop sections receiving immune secretions and those receiving nonimmune secretions.

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The Neutrophil-Activating Protein (NAP-1) Is Also Chemotactic for T Lymphocytes

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T lymphocyte chemotactic factor (TCF) was purified to homogeneity from the conditioned media of phytohemagglutinin-stimulated human blood mononuclear leukocytes by a sequence of chromatography procedures. The amino-terminal amino acid sequence of the purified TCF showed identity with neutrophil-activating protein (NAP-1). Both TCF and recombinant NAP-1 (rNAP-1) were chemotactic for neutrophils and T lymphocytes in vitro supporting the identity of TCF with NAP-1. Injection of rNAP-1 into lymphatic drainage areas of lymph nodes in Fisher rats caused accelerated emigration of only lymphocytes in high endothelial venules. Intradermal injection of rNAP-1 caused dose-dependent accumulation of neutrophils and lymphocytes.

DIRECTED MIGRATION OF LEUKOcytes along chemical gradients is fundamental to development of lymphatic tissues (*1*), lymphocyte recirculation (*2*), and accumulation of leukocytes at sites of inflammation or tissue injury (*3*). The kinetics and the site specificity of lymphocyte infiltration, as in delayed type hypersensitivity (DTH) skin lesions (*4*), suggest that leukocyte emigration may be directed by local cellular release of cytokines. We describe the purification and characterization of an 8-kD basic heparin-binding T cell chemotactic polypeptide from the conditioned media of phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) (*5*). This T cell chemotactic factor (TCF) appears to be biologically and biochemically identical to a neutrophil-chemotactic factor that is also a neutrophil-activating protein (NAP-1) that we purified and molecularly cloned (*6, 7*).

TCF was purified by sequential chromatography on heparin Sepharose (*8*), carboxymethylation exchange high-performance liquid chromatography (CM-HPLC) (*9*), and reversed-phase HPLC (*10*). To assess T cell chemotactic activity, we adapted a Boyden chamber assay (*11, 12*). The CM-HPLC fractions (fractions 52 and 53) with lymphocyte chemoattractant activity at 0.4*M* NaCl were pooled and applied to a reversed-phase HPLC column with high activity recovered in fractions 47 and 48, corresponding to a major absorbance peak that eluted at 47 min (Fig. 1). We confirmed the purity of TCF (fraction 47) by SDS–polyacrylamide gel electrophoresis (PAGE) analysis (*13*). Under reducing conditions, a single band of about 8 kD was detected. We consistently ($n = 3$) obtained pure TCF, with a yield corresponding to about 10 μg of TCF per liter of conditioned

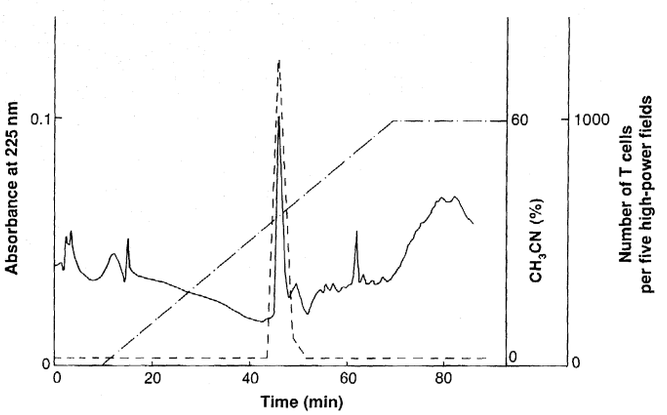

Fig. 1. T cell chemotactic activity purified from conditioned media of PHA-stimulated human PBMC by reversed-phase HPLC (*9, 12*). Absorbance is shown by the solid line; chemotactic activity, dashed line; and acetonitrile gradient, dashed and dotted line.

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medium from PHA-stimulated PBMC. We determined the NH_2-terminal amino acid sequence of TCF as described (*7*) for 13 of the first 17 amino acids (NH_2-SAKELRXQXIKXYSXPF. . .) and found that it was identical with that of NAP-1 (NH_2-SAKELRCQCIKTYSKPF. . .), which was purified from lipopolysaccharide-stimulated human monocytes (*6, 7*). The mobility of recombinant NAP-1 (rNAP-1) (*14*) was identical with TCF on SDS-PAGE.

The biological characteristics of TCF and rNAP-1 were also compared (*12*). Both TCF (fraction 47 from Fig. 1) and rNAP-1 had T cell and neutrophil chemotactic activity (Fig. 2, A and B) that diminished at higher concentrations, possibly owing to saturation of the receptors (*15*). There was no significant difference in the specific activity of TCF and rNAP-1. The maximal migration of T cells was increased an average of 7.4 times ($n = 5$) and that of neutrophils was increased 8.1 times ($n = 5$) over that of uninduced control samples. Neutrophil chemotactic activity was maximal at TCF/rNAP-1 concentrations of 10^{-2} μg/ml, and T cell chemotactic activity was maximal at 10^{-3} μg/ml. Significant T cell chemotactic activity was detected at 5×10^{-5} μg/ml and neutrophil chemotaxis at 10^{-4} μg/ml. Thus, T lymphocytes were two to ten times more sensitive to the cytokine than were neutrophils. We consistently ($n = 3$) observed, by checkerboard analysis, that T cell migration by this cytokine was dependent on a concentration gradient (chemotaxis). Less than 10% of the migration could be based on the stimulation of random migration (chemokinesis). Only 8 to 10% of the T cell–enriched cell suspension (>95% T cells) actually migrated, but all migrating cells (>99%) were small lymphocytes, as detected by light microscopy. TCF did not induce migration of cells enriched in large granular lympocytes (>70%). We confirmed that rNAP-1 did not have monocyte chemotactic activity by using a modified Boyden chamber technique (*6, 11*). Flow cytofluorometry analysis indicated that the proportion of $CD4^+$ and $CD8^+$ migrating T cells was the same as that of the input cell population. The T cell–enriched cell suspension expressed a significant number of TCF/NAP-1 receptors, but the average receptor number per cell was low compared to that of neutrophils (*16*). It is possible that only a small subset of the T cells express TCF/NAP-1 receptors. Neither TCF nor rNAP-1 stimulated the proliferation of T cells, and neither showed comitogenic effect on thymocytes with various doses of PHA, thus confirming an earlier study in which purified natural NAP-1 was used (*6*).

Intraperitoneal or subcutaneous injections of NAP-1 result in a neutrophil-dominated inflammatory response (*17*). To study the in vivo effects of rNAP-1 on lymphocytes, we injected rNAP-1 into the lymphatic drainage areas of lymph nodes in Fisher rats (*18*) and used a double-blind protocol. Injection of 0.1 ml of rNAP-1 (100 ng/ml) caused an increase in regional lymph node weight at 3, 6, and 18 hours after inoculation. Lymphocyte migration indices of these lymph nodes indicated an increase in lymphocyte traffic as early as 3 min after inoculation, with a peak of nearly three times normal traffic at 30 min, decreasing to two times normal traffic between 6 and 18 hours. The effective in vivo dose plateaued between 0.01 and 1 μg (Fig. 3A). There was no significant difference in migration across high endothelial venules (HEV) in lymph nodes draining an untreated area or those draining phosphate-buffered saline (PBS)–injected areas. However, rNAP-1 did not induce neutrophil migration across HEV. The mechanism of exclusion of neutrophils in HEV of resting lymph nodes is unknown.

We also injected rNAP-1 into ear skin of four Fisher rats (*19*) and again used a double-blind protocol. There was a significant dose-related accumulation of polymorphonuclear cells and lymphocytes ($P < 0.001$), but not monocytes in the edematous connective tissue at sites of injection (Fig. 3B). Lower doses of rNAP-1 (0.001 μg/ml) selectively attracted lymphocytes ($P < 0.009$), whereas higher doses (0.1 μg/ml) predominantly recruited neutrophils ($P < 0.0001$). Neutrophils accumulated in great numbers at the needle puncture site at all doses (Fig. 3B).

The in vivo pathophysiological relevance of TCF/NAP-1 needs to be established, but TCF/NAP-1 may play a role in the development of inflammatory reactions such as cutaneous DTH reactions. We have recently observed a considerable and rapid induction (30 min) of mRNA for NAP-1 and release of NAP-1–like activity from cultured normal human skin fibroblasts stimulated with human interleukin-1α (IL-1α) or tumor necrosis factor-α (*20*). Human keratinocytes produce IL-1 (*21*), and significant increases in IL-1–like activity (*22*), as well as the presence of a T cell chemotactic factor different from IL-1 or IL-2 (*22, 23*), appear in extracts of human epidermis overlying cutaneous DTH reactions (*24*). Furthermore, in an experimentally induced DTH-reaction (*4*), T cells infiltrate epidermis and dermis in high numbers within the first 8 hours and are followed by considerably smaller numbers of neutrophils. Perhaps IL-1, produced and released early from keratinocytes overlying a DTH reaction, induces an early local production of TCF/NAP-1, which mobilizes T lymphocytes that initiate immunologically mediated inflammatory responses. If so, this suggests that TCF/NAP-1 participates in a mediator cascade which contributes to the mobilization of inflammatory cells. Although how TCF/NAP-1 activates lymphocytes is not clear, it may stimulate the expression of various adhesive protein

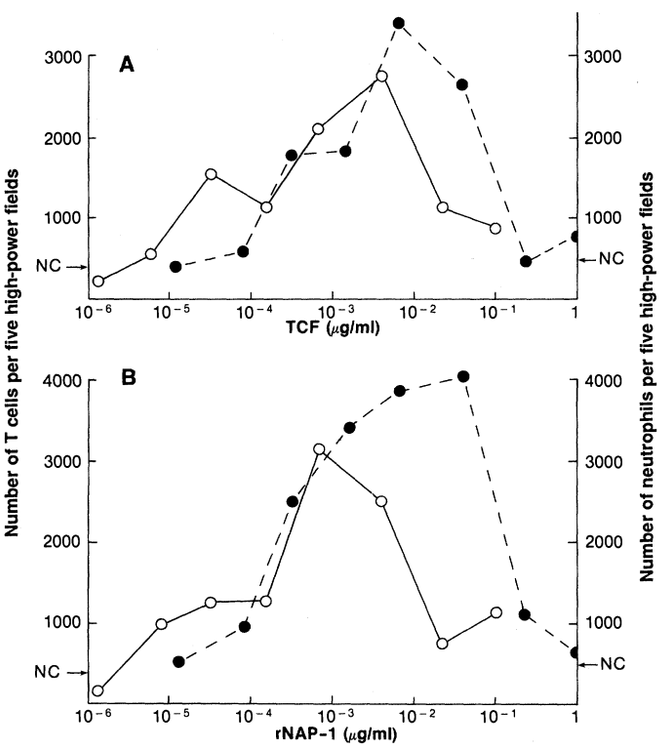

Fig. 2. Chemotactic activity, as assayed by modified Boyden chamber method, of (**A**) purified TCF and (**B**) rNAP-1 for human T cells (solid line) and neutrophils (dashed line). NC indicates levels of negative control. Pure TCF from reversed-phase HPLC was used for this assay. This experiment is representative of three similar studies.

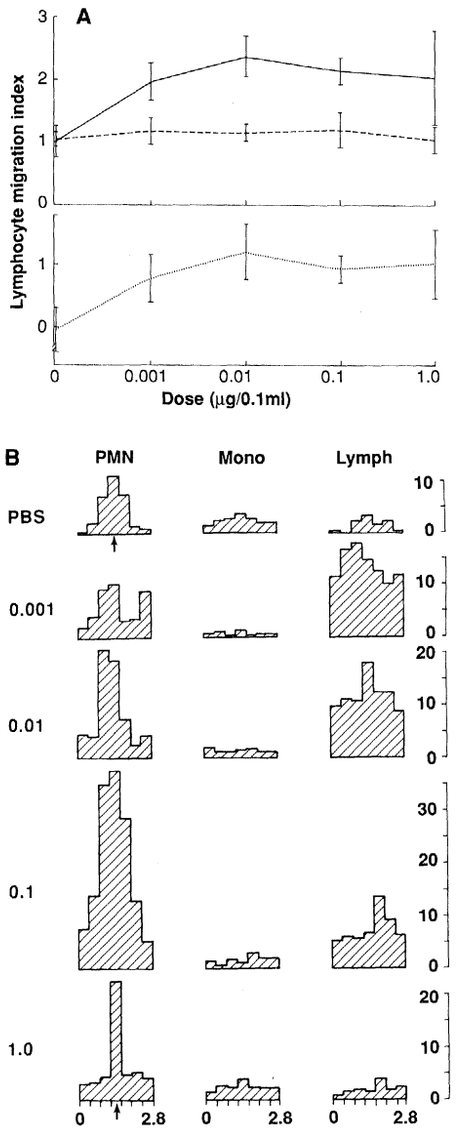

Fig. 3. (**A**) A representative study of the effects of rNAP-1 injected subcutaneously into rats. Regional lymph nodes (solid line) that drained from the subcutaneous injection sites of PBS (0.1 ml) or 0.001 to 1.0 μg of rNAP-1 in PBS (0.1 ml) and contralateral lymph nodes (dashed line) that drained from sham (PBS) inoculation sites were excised 3 hours after injection and LMI was determined. The subtraction curve (dotted line) shows mean values of LMI difference in each rat. The data are means ±2 SE (n = 4 rats per dose). Except for injection of PBS alone, LMIs for regional lymph nodes were all significantly higher than contralateral at each dose level ($P < 0.001$), with the use of SAS (release 6.03) procedure GLM to analyze variance by least square means with nested effect. (**B**) Injections of 0.02 ml of rNAP-1 (0.001 to 1.0 μg/ml) in PBS or PBS (0.02 ml) into the connective tissue of the ear of four Fisher rats. Histological sections of a rectangle (2.8 mm by 0.4 mm) of rat ear were scanned at ×100 magnification. The vertical axis gives the average number of counted polymorphonuclear leukocytes (PMN), monocytes (mono), and lymphocytes (lymph) present in each of three high-power fields on either side of a central field containing the needle track (indicated for the first column by an arrow). Statistical analysis was performed as in (A).

receptors on the cell surface such as LFA-1 (*25*), thereby facilitating the adherence to endothelial cells.

In conclusion, the range of in vitro and in vivo biological activities of NAP-1 includes T cell and neutrophil chemotactic and activating functions and is thus more extensive than previously realized. Therefore, we suggest that this cytokine be termed interleukin-8.

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5. The mononuclear leukocyte fraction from Ficoll-Hypaque sedimentation was suspended in RPMI 1640 medium with fetal calf serum (FCS) (1%, HyClone). Cells were cultured overnight at 5×10^6 cells per milliliter in the presence of PHA-P (2.5 μg/ml, Wellcome, Dartford, UK) at 37°C, and cell-free medium was obtained by centrifugation. The medium was filter sterilized and stored at −20°C.
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8. Crystalline $(NH_4)_2SO_4$ was added to the medium (1.7 liters) of PHA-stimulated PBMC to attain 80% saturation. The precipitate was collected by centrifugation, dissolved in phosphate-buffered saline, dialyzed against 0.5*M* tris-HCl, *p*H 8.0, and applied to a heparin-Sepharose column equilibrated with 0.05*M* tris-HCl, *p*H 8.0. The column (1.6 cm by 4.0 cm) was washed with equilibration buffer and with buffer containing 0.1*M* NaCl; the T cell chemotactic activity was eluted with 30 ml of 0.05*M* tris-HCl, *p*H 8.0, and 0.4*M* NaCl and concentrated to 5 ml.
9. Cation exchange HPLC was performed as described (*6*). Fractions (2 ml) were collected at a flow rate of 1 ml/min, and the lymphocyte chemotactic activity present in paired fractions was assayed.
10. Fractions 52 and 53, which contained significant lymphocyte chemotactic activity, were pooled and reversed-phase HPLC was performed as described (*6*).
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12. Migration of lymphocytes was measured by modification of a multiwell chemotaxis chamber technique for determination of leukocyte chemotaxis (*11*). Lymphocytes, in contrast to neutrophils or monocytes, do not adhere to a polyvinylpyrrolidone (PVP)–free polycarbonate chemotaxis filter and will drop to the bottom of the lower chamber. If the lower surface of the filter was coated with type IV collagen (BRL) [A. M. Pilaro and T. J. Sayers, in *Natural Killer Cells*, E. W. Ades and C. Lopez, Eds. (Karger, Basel, 1988)], T cells adhered (in clusters) and showed concentration-dependent chemotactic migration. Only 1% to 3% of the total cell number drop to the bottom of the chemotaxis chamber regardless of the TCF/rNAP-1 concentration. Freshly prepared T lymphocytes were used because T cells cultured overnight showed reduced migration and were less able to adhere to the chemotaxis filter. The chemoattractant, diluted in Dulbecco's modified Eagle's medium (DMEM) with FCS (1%), was placed in the bottom of a 48-well micro chemotaxis chamber (Neuro Probe, Cabin John, MD). T cells were purified from PBMC (*5*) and depleted of adherent cells by culture on plastic dishes and by the use of a nylon wool column [M. H. Julius, E. Simpson, L. A. Herzenberg, *Eur. J. Immunol.* **3**, 645 (1973)]. The eluted T cell suspension was more than 95% Leu 3^+, as analyzed by flow cytofluorometry. T cells (5×10^6 per milliliter) were placed in the upper 50-μl chamber, separated from the lower chamber by a 5-μm PVP-free polycarbonate chemotaxis filter. Chambers were incubated for 2 hours at 37°C in humidified air. The filters were removed, fixed as described (*11*), and stained with Giemsa. Lymphocyte migration was measured by counting the number of cells attached to the lower surface of the filter in five high-power fields, and each concentration of chemoattractant was tested in duplicate. The migrated cells consisted of more than 99% lymphocytes as determined by the morphology of the Giemsa-stained cells.
13. Samples were electrophoretically separated on a vertical slab gel of 13% acrylamide containing urea (0.53 g/ml). Samples and molecular weight standards were mixed with equal volumes of double-strength sample buffer (20% glycerol, 6% SDS with 10% 2-mercaptoethanol), and the gel was stained with the use of a silver staining kit (RAPID-Ag-STAIN, ICN).
14. The recombinant protein, rNAP-1, which corresponds exactly to the mature human NAP-1 consisting of 72 amino acids, was expressed in *Escherichia coli* by using previously cloned NAP-1 cDNA (*6*) and purified to homogeneity (R. Furuta *et al.*, in preparation).
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18. Recombinant NAP-1 (0.001 to 1.0 μg/ml) diluted in Dulbecco's PBS was injected (0.1 ml) into the lymphatic drainage bed of the left popliteal lymph nodes of Fisher rats after anesthesia with ether. The dose given to each rat and the histological specimens were each coded by a randomized double-blind protocol. After a period (3 hours) the animals were killed by cervical dislocation. The lymph nodes were excised, stripped of fat, fixed in 10% buffered Formalin, and sectioned at 3 μm across cortical lobules. At least ten coded HEV cross sections in each lymph node were counted for determination of lymphocyte migration index (LMI) [A. O. Anderson and N. D. Anderson, *Immunology* **31**, 731 (1976)] to provide an estimate of the relative rates of entry of lymphocytes into lymph nodes from the bloodstream. LMI is calculated as the number of lymphocytes between or immediately beneath HEV endothelial cells divided by the number of endothelial cell nuclei. The number of migrating lymphocytes and associated endothelial cells were counted in more than 700 cross sections through HEV present in the regional lymph nodes that drained the lymphatic beds at the sites of the injection of cytokine. Contralateral lymph nodes given sham inoculation served as controls.
19. The central area of each left ear of four rats was inoculated intradermally with 0.02 ml of PBS or rNAP-1 (0.001 to 1.0 μg/ml) coded by randomized double-blind protocol. The right ear was sham-inoculated by needle puncture only. Each ear was removed 3 hours later, fixed in Formalin, and processed for routine histology. Hematoxylin and eosin–stained sections were examined by light microscopy for differential enumeration of leukocytes.
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