

## *Ascaris suum*-Derived Products Induce Human Neutrophil Activation via a G Protein-Coupled Receptor That Interacts with the Interleukin-8 Receptor Pathway

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**Infection with tissue-migrating helminths is frequently associated with intense granulocyte infiltrations. Several host-derived factors are known to mediate granulocyte recruitment to the tissues, but less attention has been paid to how parasite-derived products trigger this process. Parasite-derived chemotactic factors which selectively recruit granulocytes have been described, but nothing is known about which cellular receptors respond to these agents. The effect of products from the nematodes *Ascaris suum*, *Toxocara canis*, and *Anisakis simplex* on human neutrophils were studied. We monitored four parameters of activation: chemotaxis, cell polarization, intracellular Ca<sup>2+</sup> transients, and priming of superoxide anion production. Body fluids of *A. suum* (ABF) and *T. canis* (TcBF) induced strong directional migration, shape change, and intracellular Ca<sup>2+</sup> transients. ABF also primed neutrophils for production of superoxide anions. Calcium mobilization in response to *A. suum*-derived products was completely abrogated by pretreatment with pertussis toxin, implicating a classical G protein-coupled receptor mechanism in the response to ABF. Moreover, pretreatment with interleukin-8 (IL-8) completely abrogated the response to ABF, demonstrating desensitization of a common pathway. However, ABF was unable to fully desensitize the response to IL-8, and binding to CXCR1 or CXCR2 was excluded in experiments using RBL-2H3 cells transfected with the two human IL-8 receptors. Our results provide the first evidence for a direct interaction between a parasite-derived chemotactic factor and the host's chemotactic network, via a novel G protein-coupled receptor which interacts with the IL-8 receptor pathway.**

Neutrophilic and eosinophilic granulocytes have evolved in the immune system as a first line of defense against invading pathogens. Remarkable numbers of eosinophils or neutrophils infiltrate lesions caused by tissue-invading parasites, as seen, e.g., in anisakiasis (57) and schistosomiasis. A series of chemokines and low-molecular-weight attractants are known to mediate recruitment of granulocytes to the site of infection (56), but less attention has been paid to the role of parasite-derived products in inflammatory infiltration. Indeed the question of whether host innate cells bear “danger” receptors for parasite products has barely been explored. In parasitic infections, there can be phenomenal intensity and selectivity of granulocyte recruitment, such as the eosinophilic phlegmons (large granulomatous infiltrations of eosinophils with marked submucosal oedema) caused by *Anisakis simplex* (anisakiasis or eosinophilic gastroenteritis) (17, 28, 30, 31) and *Ancylostoma caninum* (eosinophilic enteritis) (52, 70). Since most of the damage caused by tissue-invading parasites can be attributed to the recruited inflammatory cells, a clear picture of the mechanisms mediating granulocyte recruitment and activation is of

pivotal importance to the understanding and management of pathology.

The intensity and selectivity of inflammatory recruitment suggest that granulocytes are not simply responding to tissue injury caused by migrating larvae, but are actively targeting or being targeted by the parasites in question. Numerous parasite-derived chemotactic factors (PDCFs) have been reported to recruit, often selectively, neutrophils (neutrophil chemotactic factors [NCFs]) or eosinophils (eosinophil chemotactic factors [ECFs]) (20, 26, 43, 46–48, 51, 67, 68). Few of those, however, have been identified and cloned (20, 45). Moreover, no study has yet addressed the nature of the host cell receptors involved in this process despite the recognized importance of innate system receptors. Here, we present the first evidence that a neutrophil chemokine or related receptor may be involved in this response. Previous studies addressing granulocyte chemotaxis induced by PDCFs used granulocyte preparations with various degrees of purity from peritoneal exudate cells of guinea pigs treated with oyster glycogen or horse serum (26, 67, 68). From these studies, it is not clear to what extent the cells used were immunologically primed or contaminating cells played a role as a secondary source of chemotactic factors.

Chemoattractants have been divided into two categories by Haines et al. (23). The main category is represented by the classical chemoattractants such as formyl-Met-Leu-Phe (fMLP), platelet-activating factor (PAF), leukotriene B<sub>4</sub> (LTB<sub>4</sub>), and C5a. The isolation of interleukin-8 (IL-8) (59) over a decade

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ago heralded a new group of chemoattractant molecules, the chemoattractant cytokines (chemokines) (4, 5, 54, 58), and their receptors (37, 42, 58, 72). Both classical chemoattractants and chemokines act on target cells through seven-transmembrane-domain receptors that are coupled to heterotrimeric G proteins (42). Their engagement by an agonist results in a panoply of possible functional cellular responses, most of which are rapid and transient, e.g., a characteristic rise in the cytosolic  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ) (4), polymerization and depolymerization of actin filaments (15, 73), generation of reactive oxygen species (16), and bioactive lipids (e.g., PAF and  $LTB_4$ ) (6, 55), priming of superoxide anion production (e.g., by PAF and tumor necrosis factor alpha [TNF- $\alpha$ ]) (19, 29), and transendothelial migration (64). The second category is represented by the so-called pure chemoattractants and includes substance P (23), fibrinopeptide B (62), transforming growth factor  $\beta$ 1 (TGF $\beta$ 1) (23, 53), and Fas ligand (FasL) (44, 61). These chemoattractants are active at extremely low concentrations (TGF $\beta$ 1 at femtomolar and FasL at pico- to nanomolar concentrations) (44, 61) and do not elicit a transient  $[Ca^{2+}]_i$  increase or any degranulation (44, 53) or superoxide anion production at any concentration (23, 62). With respect to their receptors, pure chemoattractants bind to G protein-coupled receptors (substance P) (23) as well as to different receptors such as Fas (CD95 and Apo-1), the receptor for FasL (44). In this study, we demonstrate for the first time that the previously described NCF from *Ascaris suum* (68) exerts classical-like activities on human neutrophils, since its effects on target cells include pertussis toxin (PTX)-sensitive  $Ca^{2+}$  mobilization, shape change, priming of superoxide anion production, and in vitro chemotaxis.

#### MATERIALS AND METHODS

**Parasites and extracts.** Parasite body fluids were obtained from the adult stages of *A. suum* (*Ascaris* body fluid [ABF]) and *Toxocara canis* (*T. canis* body fluid [TcBF]) from naturally infected pigs and dogs, respectively. Body fluid was collected by an incision in the cuticle. The body fluids were then microcentrifuged at  $16,000 \times g$  at  $4^\circ C$  for 15 min, and the supernatants were stored at  $-70^\circ C$ . Third-stage larvae of *Anisakis simplex* were obtained by dissection of fresh, unfrozen mackerel (*Scorpaenopsis scorpaenoides*) or herring (*Clupea harengus*) from the North Sea. Somatic extracts of *A. simplex* (AnX) were obtained by snap-freezing the larvae in liquid nitrogen and grinding them to a fine powder in a mortar; 10 g of powder was extracted with 20 ml of phosphate-buffered saline (PBS, pH 7.2) at room temperature for 30 min. The extracts were then microcentrifuged at  $16,000 \times g$  at  $4^\circ C$  for 30 min, and the supernatants were stored at  $-70^\circ C$ . All protein concentrations were determined with the Coomassie Plus protein assay reagent (Pierce, Rockford, Ill.) as directed.

**Purification of granulocytes.** Neutrophils or eosinophils were purified from freshly drawn peripheral blood of healthy donors (71). Briefly, granulocytes were obtained via a two-stage protocol consisting of dextran sedimentation and a three-step isotonic discontinuous Percoll gradient (55, 70, and 81% centrifugation). The cells derived from the 55 to 70% interface were 95 to 99% neutrophils. Eosinophils were purified from the neutrophil-rich interface by a negative immunomagnetic selection step using sheep anti-mouse immunoglobulin G Dynabeads, coated with murine anti-CD16 (3G8; kind gift of J. Unkeless, Mount Sinai Medical School, New York, N.Y.). Final purities were 95 to 99% for neutrophils and  $>99\%$  for eosinophils, as assessed by cytopins stained with Diff-Quik (Dade Diagnostika, Unterschleissheim, Germany) according to the manufacturer's instructions. Viability (trypan blue dye exclusion) was at least 99% for both cell types. All buffers used during purification were  $Ca^{2+}$ - and  $Mg^{2+}$ -free.

**Chemotaxis experiments.** Chemotaxis assays were performed in a 96-well modified Boyden chamber (Neuro Probe, Gaithersburg, Md.). Positive and negative controls in the bottom wells as well as the granulocyte suspension in the upper wells were in Hanks' balanced salt solution (HBSS) (Gibco, Paisley, United Kingdom) with 0.3% bovine serum albumin (BSA) (Sigma, Poole, United

Kingdom). The polyvinylpyrrolidone-free polycarbonate membranes (Neuro Probe) had a 3- $\mu m$  pore diameter. As pointed out by Wilkinson (74), a source of error with this technique is the potential aggregation of cells during migration, resulting in adhesion to the lower surface of the filter rather than to the bottom wells. Therefore, both the cells adhering to the lower surface of the filter and the cells migrating to the bottom wells were measured as described below. Neutrophil suspension (200  $\mu l$ ,  $10^7/ml$ ) was added to the top wells, and the chemotaxis chamber was incubated at  $37^\circ C$  in humidified air with 5%  $CO_2$  for 45 min. The chemotaxis chamber was then carefully disassembled, and the upper side of the filter was washed with PBS, scraped with a cell scraper to remove adherent cells, fixed with methanol, and stained with Diff-Quik. The stained filters were measured in a 96-well spectrophotometer at 550 nm. The cells that had migrated into the bottom wells were counted in five high-power fields with a light microscope in a modified Neubauer hemacytometer. All treatments in each chemotaxis experiment were performed in triplicate. Chemotaxis was expressed as the chemotactic index (C.I.), defined as the number of cells migrating in the presence of the sample divided by the number of cells migrating in the presence of medium only.

**Measurement of cytosolic  $Ca^{2+}$  concentration.**  $Ca^{2+}$  mobilization was measured with Fura-2 according to the following protocol. Purified neutrophils or eosinophils were resuspended at a density of  $10^7/ml$  in  $Ca^{2+}$ - $Mg^{2+}$ -free HBSS-0.3% BSA without phenol red and incubated in a waterbath at  $37^\circ C$  (for all RBL-2H3 experiments at room temperature) in the presence of 2  $\mu M$  Fura-2 AM (Molecular Probes, Eugene, Oreg.), followed by two washes in the above medium. Stable transfectant RBL-2H3 cell lines expressing the human CXCR1 or CXCR2 receptor (generously provided by Ingrid Schraufstatter, La Jolla Institute for Experimental Medicine) were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Sigma), penicillin (100 U/ml), streptomycin, (100  $\mu g/ml$ ), gentamicin (50  $\mu g/ml$ ), and G418 (0.5 mg/ml) (Promega, Madison, Wis.). The Fura-2-loaded cells were then resuspended at a density of  $2 \times 10^6/ml$  in optical methacrylate (PMMA) disposable cuvettes (Kartell; Merck, Poole, United Kingdom) in 2.5 ml of HBSS-0.3% BSA with  $Ca^{2+}$  and  $Mg^{2+}$ .  $Ca^{2+}$  mobilization into the cytosol was monitored at 340 and 380 nm (excitation) and 510 nm (emission) with a spectrofluorimeter (FluoroMax; Spex Industries, Edison, N.J.) using the dm3000 Software (Spex Industries).  $Ca^{2+}$  concentrations were calculated using the Grynkiewicz equation (22). For PTX inhibition experiments, purified cells were incubated for 90 min at  $37^\circ C$  at a density of  $10^7/ml$  in HBSS-0.3% BSA in the presence of PTX (2  $\mu g/ml$ ) (Alexis Corp., San Diego, Calif.) before loading with Fura-2.

**Measurement of neutrophil polarization.** Shape change was assessed using a modification of the method described by Kitchen et al. (34): 90  $\mu l$  of a  $10^6/ml$  suspension of neutrophils in HBSS-0.3% BSA was incubated at  $37^\circ C$  in a shaking waterbath for 15 min in the presence of 10  $\mu l$  of buffer or sample. The cells were then fixed by adding 100  $\mu l$  of 2.5% glutaraldehyde in saline. Samples were analyzed for shape change by flow cytometry (EPICS Profile II; Coulter Electronics, Luton, United Kingdom). Percent shape change was determined by analyzing the whole cell population and gating on the mean forward light scatter of the non-shape-changed cells.

**Superoxide anion production and priming experiments.** Superoxide anion production of neutrophils was assessed with dihydrorhodamine 123 (DHR123) as described by Stocks et al. (65). Cells were incubated at a density of  $10^6/ml$  in HBSS-0.3% BSA with 1  $\mu M$  DHR123 (Molecular Probes) for 5 min at  $37^\circ C$  before adding a stimulus or buffer. The cells were then incubated for 12 min at  $37^\circ C$  in a shaking waterbath and immediately placed on ice. For the priming experiments, cells were incubated for 10 min at  $37^\circ C$  prior to incubation with DHR123. Superoxide anion production was analyzed by flow cytometry (EPICS Profile II) by detecting fluorescence in the green channel due to the conversion of DHR123. Data were expressed as mean fluorescence intensity of the totality of cells in the sample. For the priming experiments, any potential lipopolysaccharide (LPS) contaminants in ABF were removed with polymyxin B-Sepharose (Detoxi-Gel AffinityPak Prepacked columns; Pierce and Warriner, Cheshire, United Kingdom) according to the manufacturer's instructions.

**S-300HR gel filtration.** ABF (1 ml) was spun at  $16,000 \times g$  in a microcentrifuge for 10 min and applied at 5 mg/ml with 5% glycerol to the column (flow rate, 0.33 ml/min). Following the elution of the void volume, 40 to 45 fractions (2 ml/fraction) were collected. Protein eluting from the column was monitored by absorbance at 280 nm.

**Statistics.** All data are expressed as mean  $\pm$  standard deviation (SD) of  $n$  separate experiments, performed in duplicate or triplicate. The paired  $t$  test was used for comparison of different treatments, and  $P < 0.05$  was considered significant.

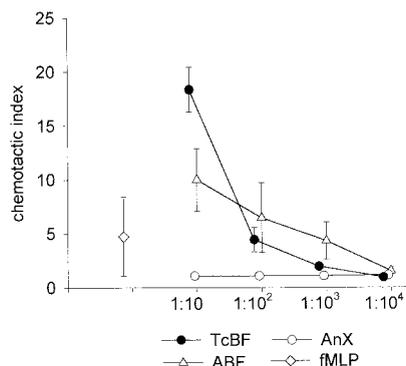


FIG. 1. In vitro chemotaxis of human neutrophils induced by products of ascarid nematodes. TcBF and ABF but not AnX, induced strong and dose-dependent granulocyte migration. Isolated neutrophils ( $10^7/\text{ml}$ ) were added to the top wells of a multiwell chemotaxis chamber, and serial dilutions were added to the bottom wells. Protein concentrations were  $500 \mu\text{g}/\text{ml}$  (1:10) to  $500 \text{ ng}/\text{ml}$  ( $1:10^4$ ) for TcBF and ABF and  $1,000 \mu\text{g}/\text{ml}$  (1:10) to  $1,000 \text{ ng}/\text{ml}$  ( $1:10^4$ ) for AnX. fMLP was used at  $10 \text{ nM}$ , as higher concentrations led to strong aggregation of the neutrophils in the filter concurrent with a loss of migration to the bottom wells. Values represent the mean  $\pm$  SD of three separate experiments with different donors performed in triplicate. SD values falling within the symbols are not shown.

## RESULTS

*A. suum* and *T. canis* but not *A. simplex* induce a strong chemotactic response in human neutrophils. The induction of chemotaxis in unprimed, high-purity populations of neutrophils obtained from peripheral blood of healthy human donors was tested using a 96-well chemotaxis chamber, and the extent of migration was assessed by counting the cells that migrated to the bottom wells. ABF and TcBF both induced a strong dose-dependent chemotactic response within the protein concentration range from  $500 \mu\text{g}/\text{ml}$  to  $500 \text{ ng}/\text{ml}$  (Fig. 1). Checkerboard titration experiments with ABF confirmed that the enhanced neutrophil migration was chemotactic rather than chemokinetic (data not shown). Somatic extracts of *A. simplex* (AnX), however, did not elicit a measurable chemotactic response in the same protein concentration range.

**ABF and TcBF induce transient intracellular  $\text{Ca}^{2+}$  ion elevations in neutrophils.** Since classic chemotactic factors such as C5a and chemokines induce  $[\text{Ca}^{2+}]_i$  transients upon engagement of their receptors (3, 22), we asked whether ABF and TcBF, in addition to their chemotactic effect on neutrophils, also mobilize  $\text{Ca}^{2+}$  ions in Fura-2-loaded cells. As shown in Fig. 2, both ABF and TcBF induced a rapid, strong, and transient elevation of  $[\text{Ca}^{2+}]_i$  in neutrophils when used at  $50 \mu\text{g}/\text{ml}$ . The intensity of the  $\text{Ca}^{2+}$  mobilization was dose dependent and still detectable with ABF diluted  $1:10^4$  (total protein concentration of  $5 \mu\text{g}/\text{ml}$ ; data not shown). ABF also mobilized  $\text{Ca}^{2+}$  in purified peripheral blood eosinophils, although more weakly (data not shown), and in the human monocytic cell line THP-1, which is known to bear one or more IL-8 receptors (data not shown). Consistent with its lack of chemotactic activity on neutrophils, AnX did not increase  $[\text{Ca}^{2+}]_i$  up to a protein concentration of  $250 \mu\text{g}/\text{ml}$ .

**ABF and TcBF induce rapid and reversible shape change in neutrophils.** A very dramatic effect elicited in target cells upon binding of chemotactic factors to their receptors is the phe-

nomenon of cell polarization. Within a few minutes, a cell that has encountered an appropriate chemotactic stimulus changes from a spherical to a highly polarized morphology (24). The shape change can be visualized under a light microscope (10) or more conveniently assessed by recording the increase in the forward angle light scatter in a flow cytometer (10, 63). Figures 3A to D show representative flow cytometry histograms of untreated neutrophils (A and B) or neutrophils treated with  $100 \text{ nM}$  fMLP (C) or ABF at  $50 \mu\text{g}/\text{ml}$  (D) for 15 min, demonstrating the strong shape change of neutrophils incubated with parasite products. Figure 3E shows the effect of serial dilutions of ABF, TcBF, and AnX on the shape of neutrophils, expressed as the percentage of cells affected. A nonchemotactic protein (chicken ovalbumin) was included to control for nonspecific effects of high protein concentrations. Neither

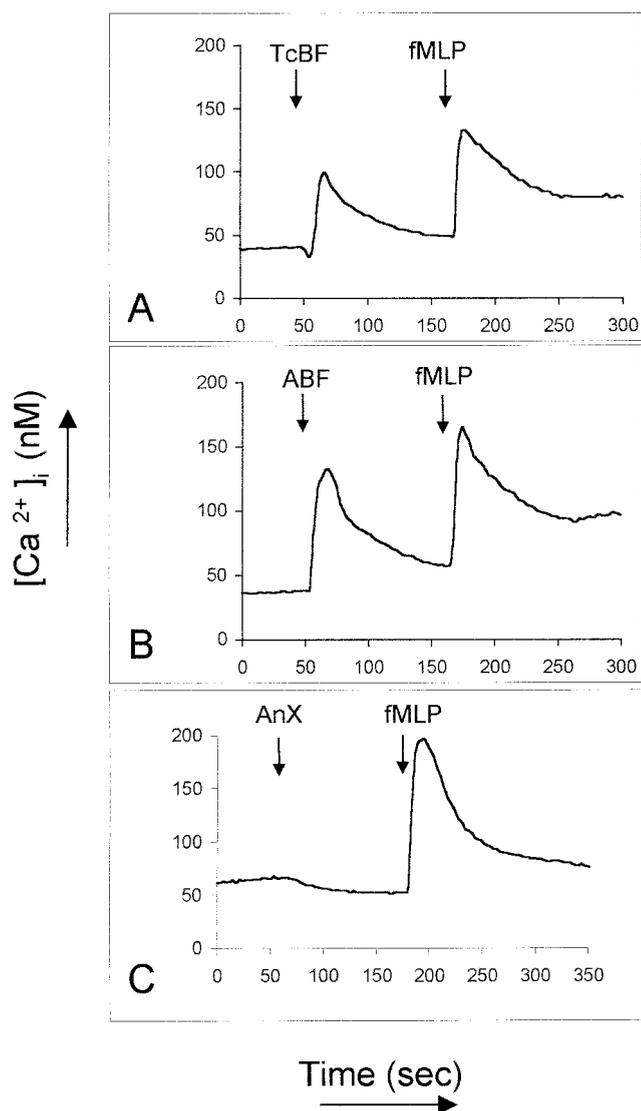


FIG. 2. Mobilization of  $[\text{Ca}^{2+}]_i$  in human neutrophils by products of ascarid nematodes. TcBF and ABF ( $50 \mu\text{g}/\text{ml}$ ) but not AnX ( $250 \mu\text{g}/\text{ml}$ ) induced rapid and strong  $\text{Ca}^{2+}$  transients in the cytosol of purified neutrophils. Each experiment was repeated with at least three different donors with comparable results.

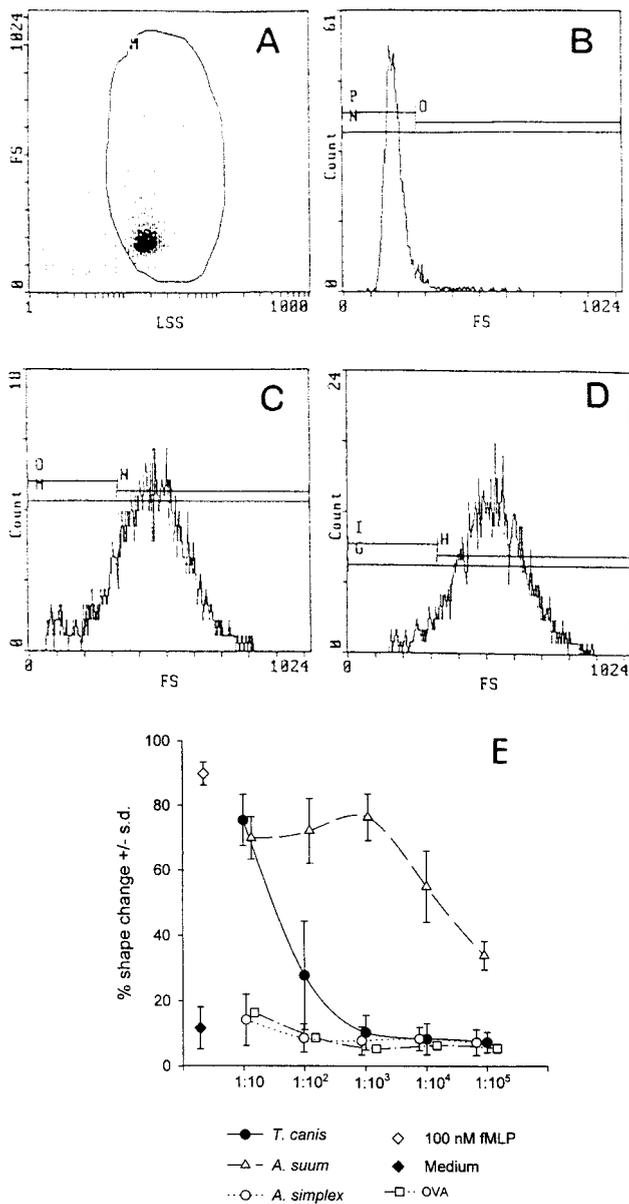


FIG. 3. Flow cytometry histograms illustrating the shape change of unprimed isolated neutrophils induced by different samples. Neutrophils were incubated with the stimuli at 37°C and fixed with 2.5% glutaraldehyde after 15 min. The autofluorescence or forward scatter of the cells was monitored on a Coulter EPICS II flow cytometer; plots show forward scatter (FS) against log side scatter (LSS). (A and B) Unprimed, unstimulated neutrophils; (C) ABF (500 μg/ml); (D) 100 nM fMLP. (E) ABF and TcBF but not AnX induce strong and reversible shape change in neutrophils. Dose-response curves obtained with the parasite products and controls. Shown are the results of three independent experiments performed in duplicate with different donors, expressed as mean percentage of cells that have undergone shape change ± SD. Also shown are 100 nM fMLP and the background shape change of unstimulated neutrophils. Protein concentrations were: ABF, TcBF, and AnX, 500 μg/ml (1:10) to 50 ng/ml (1:10<sup>5</sup>); ovalbumin (OVA), 5 mg/ml (1:10) to 500 ng/ml (1:10<sup>5</sup>).

ovalbumin (from 5 mg/ml to 50 ng/ml) nor AnX had a significant effect on the shape of neutrophils, whereas ABF and TcBF caused strong and dose-dependent shape change comparable to the intensity caused by 100 nM fMLP. ABF-induced

shape change could be detected with dilutions of up to 1:10<sup>6</sup> (corresponding to approximately 50 ng of total protein per ml) but was only detectable at higher concentrations with TcBF (500 and 50 μg/ml). Shape change induced by ABF was rapid, beginning within seconds and peaking within 5 to 15 min, depending on the donor, and reverting to basal level within 30 to 60 min (data not shown). This profile is very similar to the time course of shape change elicited by PAF (34) and IL-8 (F. H. Falcone and A. G. Rossi, unpublished data) and clearly different from the time course of shape change caused by, e.g., TNF-α or LPS (34).

**ABF effectively primes neutrophils for fMLP-induced superoxide production.** Several factors, such as IL-8, are known to induce an oxidative burst in neutrophils, evident in the production of superoxide anion (32). The induction of the oxidative burst, however, depends on the state of preactivation (priming) of the neutrophils by inflammatory factors such as PAF, TNF-α, and LPS (49). While these factors do not themselves stimulate an oxidative burst, they considerably increase this response upon a second stimulation, e.g., with fMLP (19, 29). We therefore asked whether ABF, in addition to causing Ca<sup>2+</sup> mobilization, shape change, and directional movement of neutrophils, can also prime neutrophils for enhanced superoxide anion production. Figure 4 shows the results of the oxidative burst analysis with DHR123. Treatment of neutrophils with 10 nM PAF or ABF (50 μg/ml) led to only a slight increase in superoxide anion production which was not significantly higher than in the unstimulated control. fMLP at 10 nM led to a 2.9-fold increase. Priming with 10 nM PAF 15 min prior to exposure to 10 nM fMLP led to a ninefold increase over the spontaneous oxidative activity. A similar increase (10.2-fold) was obtained when neutrophils were preincubated with ABF (50 μg/ml), showing that ABF, without inducing an oxidative burst itself, can efficiently prime neutrophils for superoxide anion production. ABF retained its priming efficiency after treatment with a polymyxin B-Sepharose column, excluding putative contamination with LPS as a possible source of priming (data not shown). Maximal (92-fold) increase was obtained with 100 nM phorbol myristate acetate (PMA) (not shown).

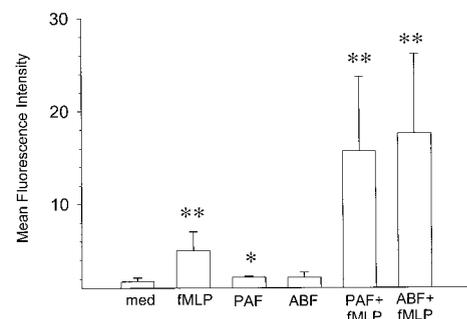


FIG. 4. ABF primes purified neutrophils for superoxide anion production. Cells were incubated at 37°C with the putative priming factors or buffer (med) for 10 min, loaded for 5 min with DHR123, and incubated for 12 min with the following stimuli: 100 nM PAF, 100 nM fMLP, ABF (500 μg/ml), or 100 nM PMA. Superoxide anion production was then measured. Values represent the mean total fluorescence ± SD for five independent duplicate determinations with different donors. \*,  $P < 0.05$ ; \*\*,  $P < 0.001$ .

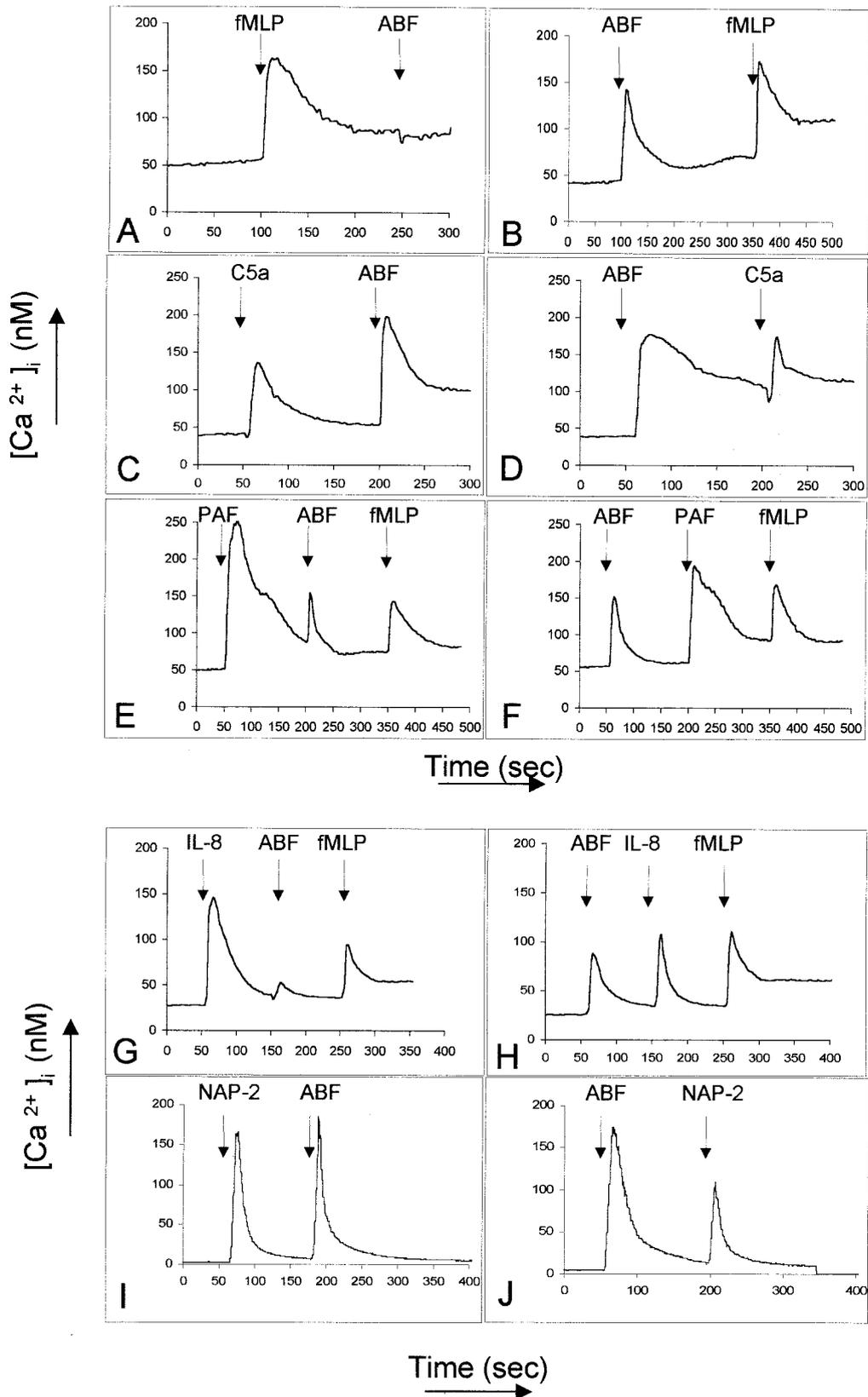


FIG. 5. Cross-desensitization experiments with ABF (50  $\mu$ g/ml) and the main chemotactic factors for neutrophils.  $Ca^{2+}$  influx was monitored with Fura-2-loaded neutrophils. (A) Desensitization of the response to ABF by 100 nM fMLP. (B) There was no cross-desensitization between 10 nM C5a and ABF (C and D), and 100 nM PAF also failed to desensitize the response to ABF as well as to 100 nM fMLP (E and F). (G) Desensitization of ABF-induced  $Ca^{2+}$  influx by IL-8. (H) Partial desensitization of the response to IL-8 by ABF. (I) Pretreatment with 100 nM NAP-2. (J) Partial (~30%) desensitization of the response to 100 nM NAP-2 by ABF. Comparable results were obtained in at least three separate experiments with different donors.

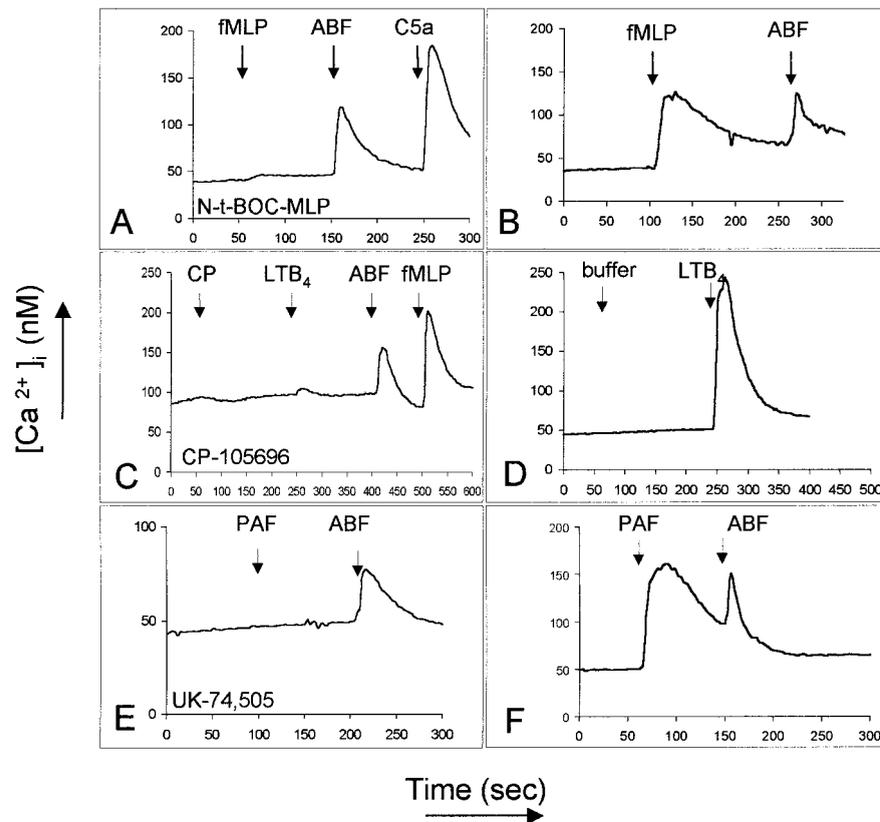


FIG. 6. Receptor-antagonist experiments with ABF (50  $\mu\text{g}/\text{ml}$ ) and specific antagonists of the receptors for fMLP (5 mM Nt-BOC-MLP, 5 min of incubation), LTB<sub>4</sub> (5  $\mu\text{M}$  CP-105,696, 3 min of incubation), and PAF (1  $\mu\text{M}$  UK-74,505, 5 min of incubation). These experiments were repeated at least three times with different donors and comparable results.

**fMLP and IL-8 desensitize the Ca<sup>2+</sup> mobilization induced by ABF.** Our next set of experiments were designed to characterize the putative receptor mediating the effects of ABF on neutrophils. These experiments exploit the phenomenon of receptor cross-desensitization. Homologous desensitization is caused by a decreased affinity of the receptor-ligand complex for G proteins and its subsequent internalization in an arrestin-dependent process, resulting in a lower response upon restimulation with the same stimulus (3). Heterologous desensitization is not dependent on receptor internalization and occurs when a receptor loses responsiveness as a consequence of a ligand's binding to a different receptor (3). Figure 5 illustrates different combinations of chemoattractant stimuli relevant to neutrophils with ABF. When neutrophils are first stimulated with 100 nM fMLP, the increase in [Ca<sup>2+</sup>]<sub>i</sub> in response to ABF is completely abrogated (Fig. 5A), whereas 10 nM C5a (Fig. 5C and D) and 100 nM PAF (Fig. 5E and F) do not affect the response significantly. Interestingly, 100 nM recombinant IL-8 almost totally desensitized the response to ABF (50  $\mu\text{g}/\text{ml}$ ) (Fig. 5G). ABF (50  $\mu\text{g}/\text{ml}$ ) partially desensitized the responses to 100 nM IL-8 (Fig. 5H) and 100 nM PAF (Fig. 5F) but not to 10 nM C5a (Fig. 5D) or 100 nM fMLP (Fig. 5B), and 100 nM neutrophil activation protein 2 (NAP-2), which is known to bind with high affinity to the chemokine receptor CXCR2 and to CXCR1 with 200-fold-lower affinity (36), did not desensitize the response to ABF (50  $\mu\text{g}/\text{ml}$ ) (Fig. 5I). Interestingly, pretreatment with ABF (50  $\mu\text{g}/\text{ml}$ ) led to a partial (about 30%) but consistent desensitization of the response to 100 nM NAP-2 (Fig. 5J).

When used in lower concentrations, fMLP ( $\leq 5$  nM) failed to desensitize the response to ABF (Fig. 6B). We also found that ABF and TcBF cross-desensitized each other's induction of Ca<sup>2+</sup> mobilization in neutrophils (data not shown). Taken together, the desensitization studies point to interactions between the receptors for fMLP (fMLP-R) and IL-8 (CXCR1 and CXCR2) in the Ca<sup>2+</sup> mobilization response to ABF. To further explore this possibility and to assess the potential role of other receptors, we performed a second set of experiments using specific receptor antagonists.

**Specific antagonists of fMLP-R, PAF-R, and LTB<sub>4</sub>-R do not inhibit ABF-induced Ca<sup>2+</sup> mobilization.** Figure 6 shows the results of incubation of neutrophils with specific antagonists. Neutrophils incubated with 5 mM Nt-BOC-MLP for 5 min prior to stimulation lost responsiveness to 10 nM fMLP but still responded fully to ABF (Fig. 6A and B). A 3-min incubation with 5  $\mu\text{M}$  CP-105,696 (kind gift from Henry Showell, Pfizer, Groton, Conn.), a specific antagonist of the LTB<sub>4</sub>-R (36), blocked the response to 10 nM LTB<sub>4</sub> but not to ABF or fMLP (Fig. 6C). A 5-min incubation with the PAF-R antagonist UK-74,505 (2) (kind gift of J. Parry, Pfizer, Sandwich, U.K.) at 1  $\mu\text{M}$ , shown in Fig. 6E, also failed to inhibit the response to ABF. These results exclude a role of the receptors for fMLP, PAF and LTB<sub>4</sub> in ABF-induced Ca<sup>2+</sup> mobilization. Taken together, the desensitization and receptor antagonist studies suggest that the effect of ABF on neutrophils may be mediated via one of the receptors for IL-8, i.e., CXCR1 or CXCR2.

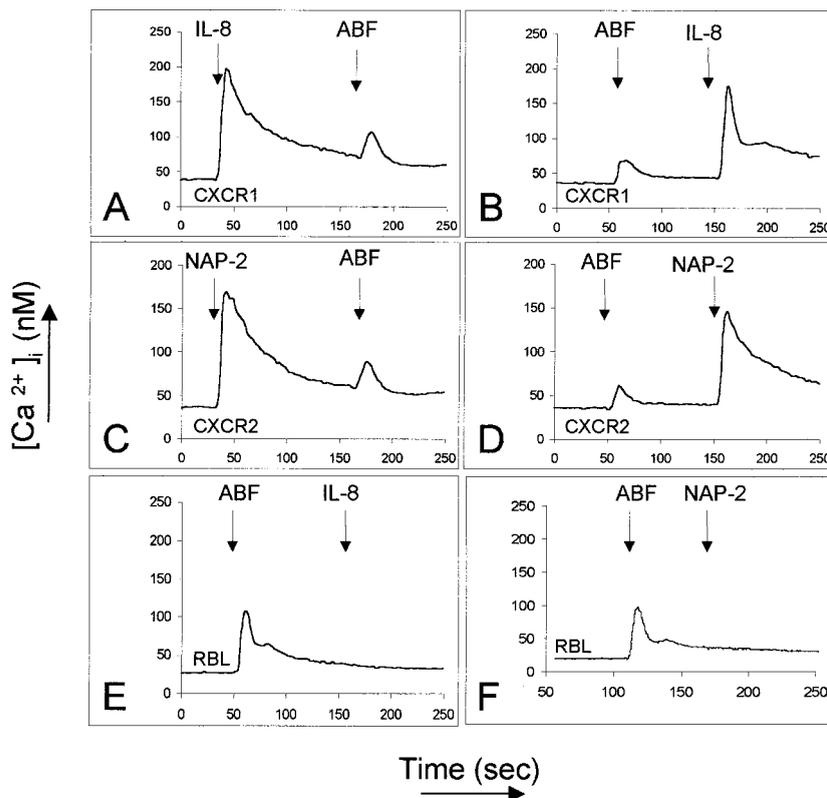


FIG. 7.  $\text{Ca}^{2+}$  mobilization by ABF in RBL-2H3 cell lines stably transfected with human CXCR1 (A and B) or CXCR2 (C and D). ABF induces a weak  $\text{Ca}^{2+}$  mobilization (via an endogenous receptor) in nontransfected as well as in transfected cell-lines (A to F), which is not desensitized by IL-8 (10 nM, A and B) or NAP-2 (100 nM, C and D). The nontransfected RBL cell line does not respond to the human chemokines (E and F). These experiments were repeated at least four times with comparable results.

**$[\text{Ca}^{2+}]_i$  transients in CXCR1 and CXCR2 transfectants are not desensitized by IL-8 or NAP-2.** To further investigate the putative role of the IL-8 receptors in *Ascaris*-dependent chemotaxis, we monitored the mobilization of intracytosolic  $\text{Ca}^{2+}$  fluxes in RBL-2H3 cells transfected with either CXCR1 or CXCR2. As shown in Fig. 7, RBL cells transfected with CXCR1 responded to IL-8 (10 nM, Fig. 7A), whereas RBL-2H3 cells transfected with CXCR2 responded to NAP-2 (100 nM, Fig. 7C). Nontransfected RBL-2H3 cells did not respond to either chemokine. Unexpectedly, nontransfected RBL-2H3 cells showed a weak but consistent response to ABF, implicating a novel, endogenous receptor present on the rat cells (Fig. 7E and F). Significantly, there was no cross-desensitization between ABF and IL-8 (Fig. 7A and B) on CXCR1 transfectants or between ABF and NAP-2 (Fig. 7C and D) on CXCR2 transfectants, excluding these receptors from being the target of the NCF in ABF on human neutrophils. In addition, it was found that extended trypsin treatment of the transfectant cell lines (45 min at 37°C) completely ablated the response to ABF but did not reduce the response to IL-8 or NAP-2 (data not shown), lending further support to the proposition that a distinct receptor may mediate *Ascaris*-dependent chemotaxis in human neutrophils.

**Inhibition of  $\text{Ca}^{2+}$  mobilization in neutrophils by PTX treatment.** Since classical chemotactic receptors are coupled to heterotrimeric, pertussis toxin-sensitive G proteins, we investigated the effect of treatment with PTX (2  $\mu\text{g}/\text{ml}$ ) on ABF-

induced mobilization of  $\text{Ca}^{2+}$ . Figure 8 shows purified neutrophils stimulated with 10 nM fMLP without (Fig. 8A) or after PTX treatment (Fig. 8B), which leads to a total inhibition of  $\text{Ca}^{2+}$  mobilization. ABF-induced  $\text{Ca}^{2+}$  mobilization (Fig. 8C) was also totally abrogated by PTX (Fig. 8D). Pretreatment with PTX, however, did not affect the influx of  $\text{Ca}^{2+}$  induced by 10 nM ionomycin, showing that the cells are still fully responsive (Fig. 8E and 8F).

**Fractionation of *Ascaris* body fluid.** Previous work performed with ABF led to the identification and biochemical characterization of separate molecular entities attracting neutrophils and eosinophils (68). Both NCF and ECF had an approximate size of 30 kDa. The neutrophil chemotactic fraction could be further separated into two distinct NCFs by isoelectric focusing (pIs of 5.2 and 7.6), which are possibly isoforms. We have performed a preliminary separation of ABF by size exclusion chromatography and tested whether, upon separation,  $\text{Ca}^{2+}$  mobilization, shape change, and chemotactic activity were coincident with a particular fraction. Activity in all three parameters was found exclusively in 5 of 45 fractions corresponding to an apparent mass ranging between 12.5 and 66 kDa (Fig. 9). These results precisely match the distribution of chemotactic activity described by Tanaka et al. (68). Since up to 50% of the protein of ABF is known to be made up by ABA-1, a retinoid and fatty acid-binding protein with a mass of 14.4 kDa (33), we asked whether ABA-1 could induce comparable immunologic effects in purified human neutrophils.

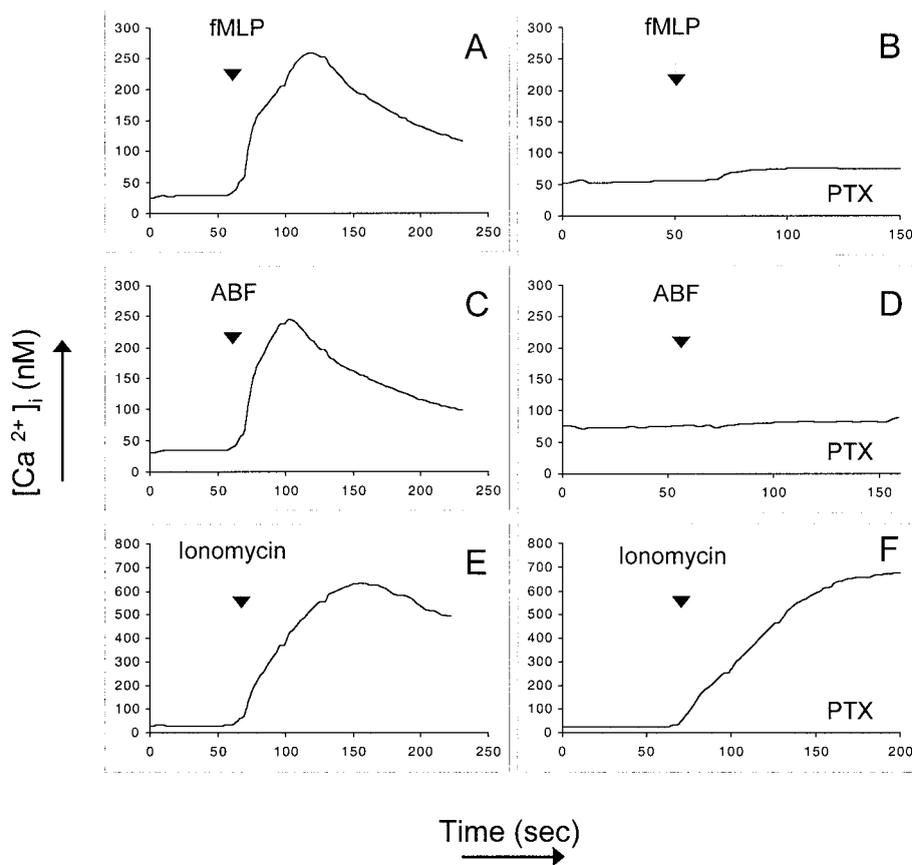


FIG. 8. Effect of pretreatment with PTX (2  $\mu\text{g}/\text{ml}$ ) on ABF-induced mobilization of  $\text{Ca}^{2+}$ . Neutrophils were stimulated with 10 nM fMLP without (A) or with PTX pretreatment (B). ABF-induced  $\text{Ca}^{2+}$  mobilization (C) was totally abrogated by PTX (D). Pretreatment with PTX did not affect the  $\text{Ca}^{2+}$  influx induced by 100 nM ionomycin (E and F). The data are representative of three experiments with the same results.

ABA-1 is also known to occur as a dimer with an approximate mass of 30 kDa and has a predicted isoelectric point of 7.55. Both physicochemical characteristics of ABA-1 are very close to the properties of one of the two NCFs described by Tanaka et al. (68). Since a homologue of ABA-1 in *Dirofilaria immitis* is chemotactic for neutrophils (11, 45) and the primary amino acid sequence of a variant of ABA-1 contains an ELR motif (41), which is well known to play an important role in neutrophil chemotaxis (25), ABA-1 is the top candidate as a putative NCF. We therefore tested parasite-derived purified (by size exclusion chromatography with fast protein liquid chromatography) and recombinant (obtained as described [39]) ABA-1 (both kindly provided by M. W. Kennedy, Glasgow, U.K.) in the  $\text{Ca}^{2+}$  mobilization and chemotaxis assays. Neither form of ABA-1 elicited  $\text{Ca}^{2+}$  mobilization (tested at 20  $\mu\text{g}/\text{ml}$ ; data not shown) in purified neutrophils or showed significant dose-dependent chemotactic activity in our in vitro assay (range, 500  $\mu\text{g}/\text{ml}$  to 500 ng/ml), except for a comparably small increase in neutrophil migration with the highest concentration of the parasite-derived ABA-1 (C.I. = 2; data not shown). The fact that only 500  $\mu\text{g}$  of purified but not recombinant ABA-1 per ml induced neutrophil chemotaxis without eliciting measurable  $\text{Ca}^{2+}$  mobilization seems to indicate that the increased migration of neutrophils was caused by contaminants of similar size (which were known to be present) in the preparation rather than by ABA-1 itself.

## DISCUSSION

We have shown that unprimed, purified human neutrophils respond rapidly to nematode body fluid constituents by in vitro chemotaxis, shape change, and  $\text{Ca}^{2+}$  mobilization. Furthermore, the most active extract, ABF, very effectively primed neutrophils for superoxide anion production.

The results of the cross-desensitization and receptor antagonist experiments indicated a possible involvement of either CXCR1 or CXCR2 in ABF-dependent neutrophil recruitment. The observed cross-desensitization by high concentrations of fMLP was not caused by homologous desensitization, since the receptor antagonist studies excluded this receptor from being the target of the NCF in ABF. Rather, fMLP is known to act as a strong (heterologous) desensitizer of  $\text{Ca}^{2+}$  mobilization induced by IL-8 (3). The desensitization of ABF-induced  $\text{Ca}^{2+}$  mobilization by 100 nM fMLP also rules out the involvement of the receptor FPRL1 as the putative receptor for the NCF in our study. FPRL1 has recently been shown to be a receptor for the acute-phase protein serum amyloid A (SAA) (66). Due to the apparently low affinity of FPRL1 for fMLP, only very high concentrations of fMLP (10  $\mu\text{M}$  to 1 mM) are able to desensitize the response to SAA (66). Since our study demonstrated a total desensitization of neutrophils to ABF with 10 or 100 nM fMLP, we think that this result rules out a possible involvement of FPRL1.

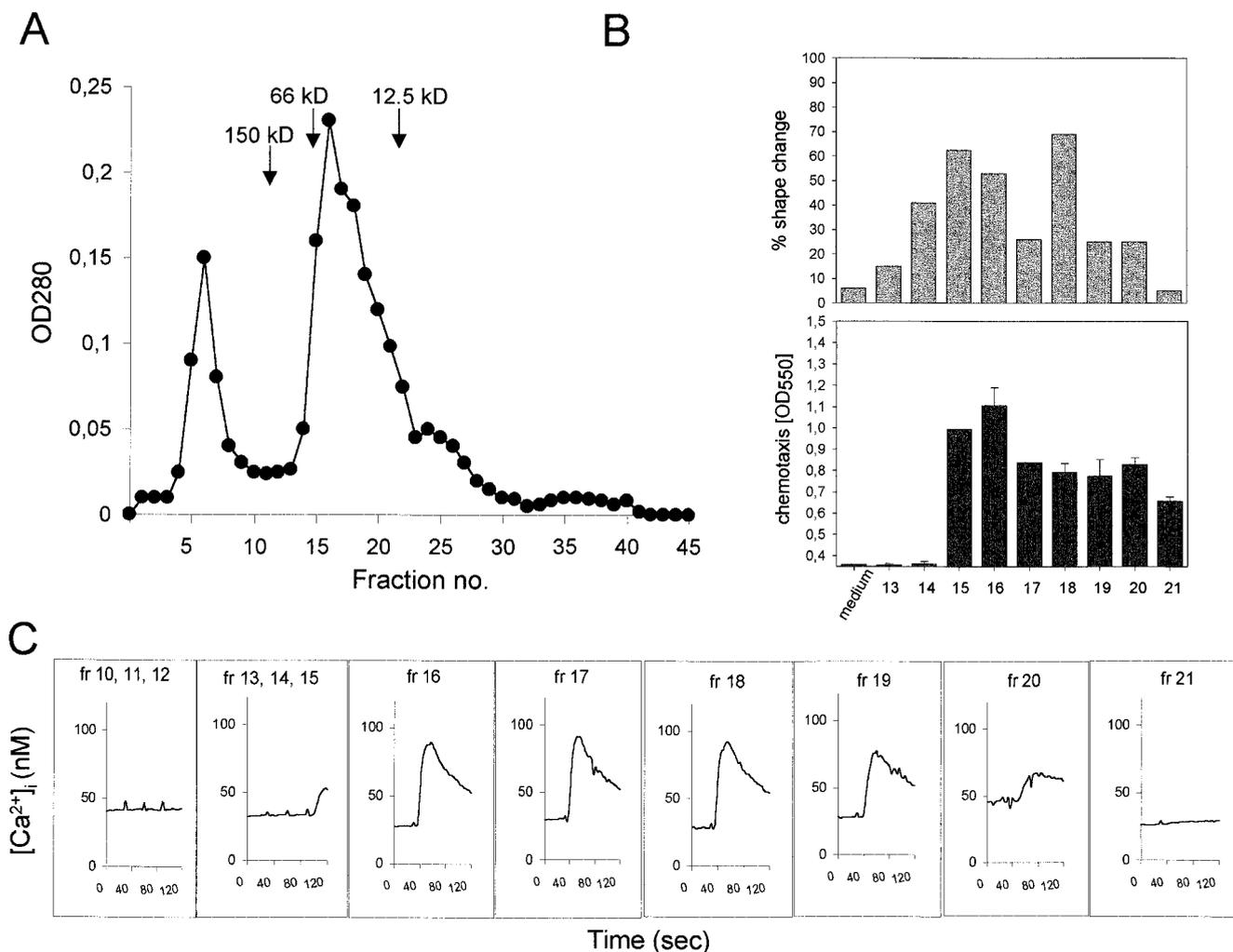


FIG. 9. Distribution of stimulatory activities in size exclusion chromatography fractions of ABF. Shown are the chromatogram with the calibration standards (A), the percent shape change (as described above), and chemotactic (obtained from the fixed and stained cells that migrated through and adhered to the bottom side of the filter) activities in the single fractions (B), and the Ca<sup>2+</sup> mobilization (C) from Fura-2-loaded cells. All three activities coincided in fractions 15 to 20. OD, optical density.

Several results led us to assume an involvement of CXCR1 or CXCR2. PAF, when used as the first signal, did not desensitize the Ca<sup>2+</sup> mobilization induced by ABF, but when ABF was given first, the response to PAF was partially desensitized. Such a partial (10 to 24%) desensitization of PAF signalling by IL-8 has been described before (3). The effective induction of Ca<sup>2+</sup> mobilization by ABF in THP-1 cells is also consistent with a role of CXCR1 or CXCR2, since a receptor for IL-8 has been described in these cells (21, 27). The Ca<sup>2+</sup> mobilization in eosinophils is better explained by the activity of the ECF rather than the NCF in ABF, since Petering et al. have recently shown that eosinophils do not express receptors for IL-8 (50). The observation that ABF did not significantly increase superoxide anion production would point to CXCR2 rather than CXCR1 as the putative target receptor, since superoxide anion production induced directly by IL-8 is mediated by CXCR1 but not CXCR2 (32). In this context, the strong effect of ABF is consistent with the known effective priming of neutrophils pretreated with IL-8 (14). Nevertheless, although all our initial results indicated a possible involvement of CXCR2 (or

CXCR1), the experiments with the transfected cell lines led us to reject this hypothesis. The strong cross-desensitization between IL-8 and ABF and the weaker (but consistent) desensitization of the neutrophil response to NAP-2, however, suggest that *A. suum* may directly affect neutrophils via a receptor that interacts with both IL-8 receptors but not with the receptors for fMLP, FPRL1, LTB<sub>4</sub>, or C5a and only weakly with the receptor for PAF. Thus, our results raise the interesting possibility that human neutrophils may express a receptor which may enable the immune system to detect, target, and ultimately destroy tissue-migrating helminth larvae. Along these lines, the observed priming for superoxide production by parasitic products such as those contained in ABF may serve to enhance the immune response to the intruding parasite or alternatively support scavenger-like functions such as the removal of damaged or dead parasites. Future work will aim to test whether secreted products can activate neutrophils by the same mechanism, or if the reaction is dependent upon body fluid components released only by dead or dying organisms.

What is the biological significance of neutrophil attraction

by parasitic products? It is widely accepted that neutrophils are of paramount importance as a first line of defense against bacteria. This is demonstrated by the hereditary disease chronic granulomatous disease (60), in which a defect in the leukocyte oxidase results in a severe deficiency in immunity to several, especially catalase-positive, pathogens (40). Neutrophils are highly responsive to fMLP and related formylated peptides. N-Formylpeptides are bacterial products, and it can therefore be assumed that the main receptor for these products, fMLP-R (7, 8) (or FPR for formylpeptide receptor), plays an important role in antibacterial defense. This view is strongly supported by a recent study by Gao et al. (18), showing that FPR<sup>-/-</sup> mice display a significantly higher mortality than their wild-type counterparts when challenged with *Listeria monocytogenes*. It is therefore likely that an analogous recognition mechanism has evolved to mediate responses to multicellular parasites, i.e., helminths. The association between helminth infection and eosinophils has been known for over a century (9), but the role of neutrophils has been less well appreciated. Indeed, the recruitment of eosinophils may itself be indirect and mediated by neutrophils. Neutrophils have the ability to release ECFs upon diverse stimuli, including parasite-derived factors (12, 13, 35, 48), and have been proposed to be mediators of eosinophil recruitment, e.g., in infection with *Schistosoma japonicum* (46).

A note of caution has to be made regarding the interpretation of our priming experiments. Bacterial endotoxin (LPS) is the classical priming agent for neutrophils (1, 49), and we cannot completely exclude the presence of minute amounts of LPS or other bacterial contaminants in ABF, causing some fraction of its priming effect. Treatment of ABF with polymyxin B-Sepharose did not affect its efficiency as a priming agent. Furthermore, priming of neutrophils by LPS is strongly dependent on the presence of mediators found exclusively in serum or plasma (49) (e.g., LPS-binding protein [69] and septicin [75]), which were absent in our assays. We therefore think that putative bacterial contaminants cannot be responsible for the strong priming of neutrophils by ABF.

In summary, we show for the first time how a PDCF contained in ABF induces not only chemotaxis but also strong activation of human neutrophils (calcium mobilization, shape change, and priming of superoxide production) in vitro. To the best of our knowledge, this is also the first report of an NCF activity in *T. canis*. Our data indicate that these effects are mediated by a receptor which interacts strongly with CXCR1 and weakly with CXCR2 and PAF-R, but is distinct from these receptors and from the receptors for LTB<sub>4</sub>, C5a, fMLP (FPR), and SAA (FPRL1). The strong and specific interaction with the IL-8 receptor pathway and the PTX sensitivity suggest that the target receptor is a member of the serpentine, heterotrimeric G protein-coupled receptor superfamily. Future work will address the molecular identity of this receptor on human neutrophils.

We expect that the techniques used in this study, in combination with the advances in parasite genome sequencing and the rapidly increasing knowledge about the chemokine network, will enable a more thorough understanding of granulocyte recruitment in helminth infections. The understanding of the underlying mechanisms, in addition to suggesting new therapeutic approaches for the treatment of acute parasite-induced

pathology, may also impact directly on our understanding of the mechanism of allergic or inflammatory diseases.

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