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HIV-induced T-cell syncytia release a two component T-helper cell chemoattractant composed of Nef and Tat

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SUMMARY

Using a newly developed gradient chamber to provide independent measurements of chemokinesis (stimulated motility) and chemotaxis (stimulated motility up a concentration gradient) of individual T-helper cells, it was recently demonstrated that HIV-induced T-cell syncytia release two distinct chemotactic activities that are separable by their rates of diffusion. The molecular masses of the two chemoattractant activities were estimated to be 30 and 120 kDa. The higher molecular mass activity was demonstrated to be the viral glycoprotein gp120. In an attempt to identify the lower molecular mass activity, chemotaxis and chemokinesis of T-helper cells were analyzed in individual concentration gradients of the virally encoded proteins Rev, p24, Tat and Nef. None functioned alone as a chemoattractant, but both Tat and Nef alone functioned as chemokinetic stimulants. When Tat and Nef were used together to generate parallel gradients, they stimulated chemotaxis. Antibody to either Tat or Nef neutralized the lower molecular mass chemotactic activity released by syncytia. The addition of antibody to the CD4 receptor or the addition of soluble CD4 inhibited high molecular mass chemotactic activity but not the low molecular mass chemotactic activity in HIV-induced syncytium-conditioned medium, demonstrating that the former but not the latter activity is mediated through the CD4 receptor. These results identify the combination of Nef and Tat as the lower molecular mass T cell chemoattractant released by HIV-induced syncytia, and provide the first evidence suggesting that parallel concentration gradients of two proteins are necessary for chemotaxis.

Key words: HIV, Nef, Tat, Two component chemoattractant, HIV-induced syncytium

INTRODUCTION

HIV-infected T-cells express the virally encoded glycoprotein gp120 on their surface (Kowolski et al., 1987; McDougal et al., 1986). Cell surface gp120 then can interact with the CD4 receptor of uninfected T-cells to initiate cell fusion and the genesis of multinucleated syncytia (Lifson et al., 1986a,b). In culture, gp120-mediated T-cell fusions can lead to syncytia many thousands of times the volume of a single cell, and can account for the majority of T-cell death (Sylwester et al., 1997). During long-term videoanalysis of HIV-infected cultures, it was found that single cells sometimes moved in a directed fashion toward syncytia. In order to identify the activity responsible for this directed movement, a chemotaxis chamber was developed that is capable of generating gradients of relatively high molecular mass molecules, and that allows continuous and direct observation of each cell as it responds to the evolving chemical gradient (Shutt et al., 1998). By continuously videorecording cells and analyzing their behavior using the computer assisted dynamic image analysis system DIAS (Soll, 1995; Soll and Voss, 1998), chemotactic stimulation and chemokinetic stimulation were independently monitored as steep gradients of test substances formed and then flattened in the chamber. Using this system, we demonstrated that HIV-induced T-cell syncytia release two separable chemotactic activities into the medium (Shutt et al., 1998).

Because the time it takes for a gradient of a molecular species to form by diffusion is a function of its Stokes radius, and therefore molecular mass (Tanford, 1961; Daniels and Alberty, 1963; Lauffenberger et al., 1988), the time it takes a cell to respond maximally to an evolving gradient in the chamber can be used as a bioassay to estimate the molecular mass of unidentified stimulants (Shutt et al., 1998). Using this bioassay, the two chemoattractants released from HIV-infected T-cell syncytia were estimated to have approximate molecular masses of 30 and 120 kDa (Shutt et al., 1998). Because 120 kDa matched the molecular mass of the virally encoded glycoprotein gp120, and because gp120 is shed by HIV-infected cells into the medium (Gelderblom et al., 1985; Schneider et al., 1986), anti-gp120 antibody was added to syncytium-conditioned medium to test whether it neutralized the higher molecular mass chemotactic activity. Anti-gp120 antibody eliminated the higher molecular mass activity, but not the lower molecular mass activity (Shutt et al., 1998). In addition, it was demonstrated that purified gp120 functioned as a T-helper cell chemoattractant both by the single cell assay

(Shutt et al., 1998), which directly assesses chemotaxis and by the Boyden chamber assay (Weissman et al., 1997; Kornfeld et al., 1988), which indirectly assesses chemotaxis. It was also demonstrated that anti-CD4 antibody neutralized the higher molecular mass chemotactic activity (Shutt et al., 1998), supporting its identity as gp120 and suggesting CD4 as part of the chemotactic receptor. The lower molecular mass chemotactic activity released by HIV-induced syncytia, however, was not identified.

Here, we have tested whether any of four low molecular mass proteins encoded by HIV, namely Rev, p24, Nef and Tat, function as T-helper cell chemoattractants, and whether any of them represent the lower molecular mass chemotactic activity released by HIV-induced T-cell syncytia. Previous studies using Boyden chamber-type filter assays indicated that Tat functioned as a chemotactic or chemokinetic stimulant of monocytes and dendritic cells (Lafrenie et al., 1996b; Mitola et al., 1997; Benelli et al., 1998). There have been no reports, however, suggesting that Rev, p24, Nef or Tat functioned as chemoattractants of T cells. In addition, reservations have been raised concerning the efficacy of Boyden chamber-type filter assays in distinguishing between chemotaxis and chemokinesis (Zigmond, 1978; Wilkinson, 1988; Zigmond and Hirsch, 1973; Rhodes, 1982; Shutt et al., 1998). We have, therefore, tested whether Rev, p24, Tat or Nef function as T cell chemoattractants using a chemotaxis chamber and a computer-assisted motion analysis system that analyze the response of all cells in a test population and that unambiguously distinguish between chemokinetic and chemotactic stimulation (Shutt et al., 1998). Evidence is presented that none of the four tested virally encoded proteins alone function as a chemoattractant. Nef and Tat, however, each function alone as chemokinetic stimulants of T-helper cells, and when simultaneous gradients of the two are established, they function in combination as a bona fide chemoattractant. Inhibition experiments with antibodies further demonstrate that Nef and Tat together represent the lower molecular mass chemoattractant released by HIV-induced syncytia, and that CD4 is not part of the chemotactic receptor for Nef or Tat.

MATERIALS AND METHODS

Culturing T-helper cells

To obtain peripheral blood T-helper cells (PBTHC) for chemotaxis assays, 120 ml of blood were drawn from healthy, HIV-negative donors into heparinized syringes and 30 ml were overlaid on 15 ml of Ficoll-Hypaque solution in each of four 50 ml conical tubes. Tubes were centrifuged for 40 minutes at 550 ***g***. The band of mononuclear cells atop each of the Ficoll-Hypaque cushions were pooled in a 50 ml conical tube, pelleted at 1000 ***g*** for 10 minutes and washed three times in Earle's balanced salts solution (EBSS). Cells were resuspended in 40 ml of RPMI 1640 medium (Life Technologies, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories, Inc., Logan, UT), 10 mM HEPES, 2 mM L-glutamine, 1× MEM non-essential amino acids, 100 units per ml of penicillin, and 100 μg per ml streptomycin sulfate (supplemented RPMI medium). Twenty ml of this cell suspension were transferred to each of two T-75 culture flasks. After 2 hours of incubation at 37°C in 5% CO_2, non-adherent cells were transferred to a 50 ml conical tube, gently pelleted and counted in a haemocytometer. This step removed the majority of monocytes. Approximately 1.25×10^8 cells were incubated with anti-CD8 and anti-CD19 antibody bound to magnetic beads, then passed through a paramagnetic column (Miltenyi Biotec, Inc., Auburn, CA) according to the manufacturer's protocol. This step depleted both CD8+ T-cells and B-cells from the culture. Flow-through cells were pelleted, then resuspended at a final concentration of 1×10^6 per ml in supplemented RPMI 1640 medium containing 20% FBS, 10 μg per ml phytohemagglutinin and 10 U per ml delectinated IL-2 (stimulation medium). This procedure resulted in final cell preparations that contained greater than 90% CD4+ PBTHCs (Shutt et al., 1995b). Before use in chemotaxis experiments, PBTHCs were grown in stimulation medium for a minimum of 72 hours. Cells of the immortalized SupT1 cell line (Smith et al., 1984), used for generating HIV-induced syncytia, were maintained according to methods previously described (Sylwester et al., 1993, 1995, 1997; Shutt et al., 1995b).

Collecting medium conditioned by HIV-induced T cell syncytia

HIV-1LAI (previously referred to as HTLV-IIIB) was maintained in cell cultures according to methods previously described (Sylwester et al., 1993, 1995, 1997; Shutt et al., 1995b). For infection, 10^7 SupT1 cells were pelleted, resuspended in 1 ml of infected culture supernatant containing 500 $TCID_{50}$ (50% tissue culture infectious doses) of HIV-1LAI and incubated for 2 hours. Cells were then diluted into fresh medium. To obtain syncytium-conditioned medium, 72 hour post-infected cultures were placed on the stage of an inverted microscope inside a biosafety cabinet and approximately 800 individual syncytia, identified by size under phase contrast at 100× magnification, were picked with a micropipette, transferred to a 1.5 ml screw cap tube containing 0.5 ml of fresh supplemented RPMI medium and incubated for 6 hours at 37°C in a 5% CO_2-air mixture. The syncytia averaged approximately 100 single cell volume equivalents (Sylwester et al., 1997), and remained intact throughout incubation. The syncytium culture was then gently centrifuged, and the supernatant removed, aliquoted and stored at −80°C prior to use in a chemotaxis experiment.

Setting up the chemotaxis chamber

The methods and the gradient chamber used to analyze single PBTHC locomotion in concentration gradients have been described in detail in a preceding publication (Shutt et al., 1998) and will only be briefly described here. The chamber consisted of a shallow channel across a bridge separating two deep chambers. Both chamber walls were removable. The chamber design was similar to that previously described (Shutt et al., 1998), but the chamber body was machined in stainless steel rather than aluminum and the bridge was made of quartz rather than glass. To initiate a chemotaxis experiment, 200 μl of 3 to 7 day cultures of PBTHCs at a density of 2×10^6 cells per ml were distributed onto the center of a 24×30 mm Thermanox plastic coverslip (Nunc, Inc., Naperville, IL) positioned inside of a plastic Petri dish. This preparation was then incubated overnight at 37°C in a 5% CO_2-air mixture. This step increases the proportion of motile cells (O'Neill and Parrott, 1977). At the end of the incubation period, excess medium was removed, and the coverslip with adhering cells was inverted and clamped to the top of an inverted chamber. The chamber was then turned right-side-up and the source and sink wells simultaneously filled with the solutions indicated for each experiment. A second coverslip was lowered onto the chamber at an angle, beginning at the sink and ending at the source to minimize contamination of the latter with the former. The following concentrations of test molecules and antibodies were used in the source well of the chamber in chemotaxis experiments: 1 μg per ml of Nef (ViroGen Corp., Watertown, MA); 1 μg per ml of p24 (cat. # 382), obtained from the AIDS Research and Reference Reagent Program of NIH (ARRRP), donor Dr K. Steimer, Chiron Corporation (Barr et al., 1987; Steimer et al., 1986); 1 μg per ml Tat (cat. # 2222), obtained from ARRRP, donor Dr J. Brady (Bohan et al., 1992); 1 μg

per ml Rev (cat. # 1457), obtained from ARRRP, donor Mr. M. Orsini, Dr D. Rekosh, Dr M.-L. Hammarskjöld; 2 μg per ml of recombinant soluble CD4 (Cat. # 1813) obtained from ARRRP, donor Dr R. Sweet (Arthos et al., 1989); 100 ng per ml of IP-10 (R & D Systems; Loetscher et al., 1996); 10 ng per ml of MIP-1α (R & D Systems; Campbell et al., 1996); 100 ng per ml of IL-8 (R & D Systems; Taub et al., 1996); 100 ng per ml SDF-1β (R & D Systems; Bleul et al., 1996); and 3×10^{-11} M IL-16, a generous gift from Dr W. W. Cruikshank, Boston University (Cruikshank et al., 1991). Antibodies for blocking experiments were used at the following concentrations: anti-Nef ascites fluid, clone AG11 (cat. # 963) at 1:50 dilution, obtained from ARRRP, donor Dr J. Hoxie (Inoue et al., 1993); anti-Tat (cat. # 1973, 1974), at 250 ng per ml, obtained from ARRRP, contributor NIAID (Campioni et al., 1995); and anti-CD4 at 4 μg/ml, a gift from Dr R. C. Kennedy, University of Oklahoma (Attanasio et al., 1991).

The analysis of cell behavior in a gradient chamber

To analyze single cell behavior over time at the bridge, the loaded chamber was immediately positioned upon the stage of an inverted microscope (Axiovert 100, Zeiss Corp., Germany) equipped with a long distance 10× objective and condenser. Fields of cells were imaged through a CCD camera (Cohu, Inc., San Diego, CA) and digitized into the Dynamic Image Analysis System (DIAS) data base (Soll, 1995; Soll and Voss, 1998; Soll, 1999) in a Macintosh 7100 Power PC computer (Apple Computer Inc., Cupertino, CA). The temperature of the chamber was maintained at 37±1°C using a custom-built thermostatically controlled stage heater. Images were digitized at 15 second intervals using a frame-grabber (Data Translations, San Diego, CA). DIAS software was used to process the images. DIAS automatically outlined the cell perimeter in each image and computed the centroid (center of area) of each cell image from the β-spline replacement image (Soll, 1995). Instantaneous velocity (I.V.) was computed by the central difference method (Maron, 1982) according to the formula $I.V.(f) = d1 + d2/2\Delta t$, where d1 and d2 are the distances in μm traveled during two sequential intervals 1 and 2 between the frame preceding f and the frame following f, respectively, and Δt is the time interval between successive frames (Soll, 1995; Soll and Voss, 1998). The chemotactic index (C.I.) was computed as the net distance traveled by a cell in the direction of the source well containing attractant, divided by the total distance traveled by the cell. A C.I. of +1.0 represents direct linear movement towards the source and a C.I. of –1.00 represents direct linear movement away from the source. A C.I. of 0.00 represents zero net movement toward or away from the source. Positive average chemotactic indices for a variety of cell types in gradient chambers range between +0.15 and +0.70 (Zigmond, 1977; Varnum-Finney et al., 1987; Shutt et al., 1995a). With the gradient chamber used in the present study, the average C.I. of PBTHCs responding to gradients of the lower and higher molecular mass chemotactic activities released by HIV-induced syncytia were previously measured to be +0.27 and +0.36, respectively (Shutt et al., 1998). A second measure of chemotactic responsiveness used in this study was the percentage of translocating cells that exhibit a positive chemotactic index (CP+). A CP+ of 50% indicates random movement, while a CP+ of 100% indicates positive chemotactic indices for all migrating cells in a population. Since a minority of PBTCs (~25%) in each preparation were not continuously motile, only cells in the population migrating at average I.V.s greater than 3.0 μm per minute over a period greater than seven minutes were analyzed for C.I. and CP+. The CP+ of PBTHCs responding to concentration gradients of the lower and higher molecular mass chemotactic activities were previously measured to be 73% and 85%, respectively (Shutt et al., 1998). Chemokinetic stimulation was computed as the average instantaneous velocity in a gradient of a test substance minus the average instantaneous velocity in buffer, divided by the average instantaneous velocity in buffer, and expressed as a percentage.

RESULTS

HIV-induced T-cell syncytia release two separable chemoattractant activities

When the single cell chemotaxis chamber was used to monitor percentage positive chemotaxis (CP+) of a population of PBTHCs responding to medium conditioned by HIV-induced T cell syncytia (syncytium-conditioned medium), two activity peaks were obtained at approximately 55 and 115 minutes (Fig. 1A). Because the time it takes for a molecule in the source well of the chamber to generate a steep concentration gradient across the bridge is proportional to its molecular mass (Tanford, 1961; Daniels and Alberty, 1963; Lauffenberger et al., 1988), the chamber also provided a bioassay for estimating the molecular masses of the two chemotactic activities in syncytium-conditioned medium. The times of maximum chemokinetic or chemotactic response were measured in the chamber for a variety of cell types responding to chemotactic and chemokinetic agents with known molecular masses (see legend of Fig. 1). The time of maximum response in each case was plotted as a function of molecular mass, and a best-fit straight line was generated (Fig. 1B). Using this standards plot (Fig. 1B), the molecular mass of the two chemotactic activities were estimated to be approximately 30 kDa and 120 kDa (Shutt et al., 1998). The higher molecular mass chemoattractant was identified as the virally encoded protein gp120 (Shutt et al., 1998), but the lower molecular mass chemotactic activity was not identified.

Individually, Nef and Tat are chemokinetic stimulants, but not chemoattractants

The absence of both the lower or higher molecular mass chemotactic activity in the medium of polyethylene glycol (PEG)-induced T-cell syncytia (Shutt et al., 1998) suggested that the two activities released by HIV-induced syncytia were either encoded for or induced by HIV (Shutt et al., 1998). Therefore, the four low molecular mass virally encoded proteins Rev, p24, Tat, and Nef were tested in the chamber to determine if they could be considered candidates for the lower molecular mass chemotactic activity released by HIV-induced T-cell syncytia. Neither Rev, a regulator of viral gene expression (Pomerantz et al., 1992; Kim et al., 1989; Klotman et al., 1991), nor p24, a capsid protein (Dorfman et al., 1994; von Poblotzki et al., 1993), functioned as either a chemotactic or a chemokinetic stimulant of PBTHCs. PBTHCs were analyzed over a period of time that exceeded the expected time it would take these molecules, based on their molecular masses, to generate a steep gradient through diffusion. In a concentration gradient of Rev, the average chemotactic index (C.I.) and the proportion of chemotactically responsive cells (CP+) were -0.06±0.46 and 36%, respectively, and in a concentration gradient of p24, the C.I. and CP+ were +0.07±0.43 and 60%, respectively (Table 1). These values were similar to the respective parameters of PBTHCs in buffer (+0.07±0.51 and 54%, respectively), but far below those for PBTHCs responding to a concentration gradient of the unknown lower molecular mass chemotactic activity released by HIV-induced syncytia (+0.31±0.49 and 78%, respectively; Table 1). The percentage chemokinetic stimulation of PBTHCs responding to concentration gradients of Rev or p24 was in each case 0%, far below the value of 49% for PBTHCs in a

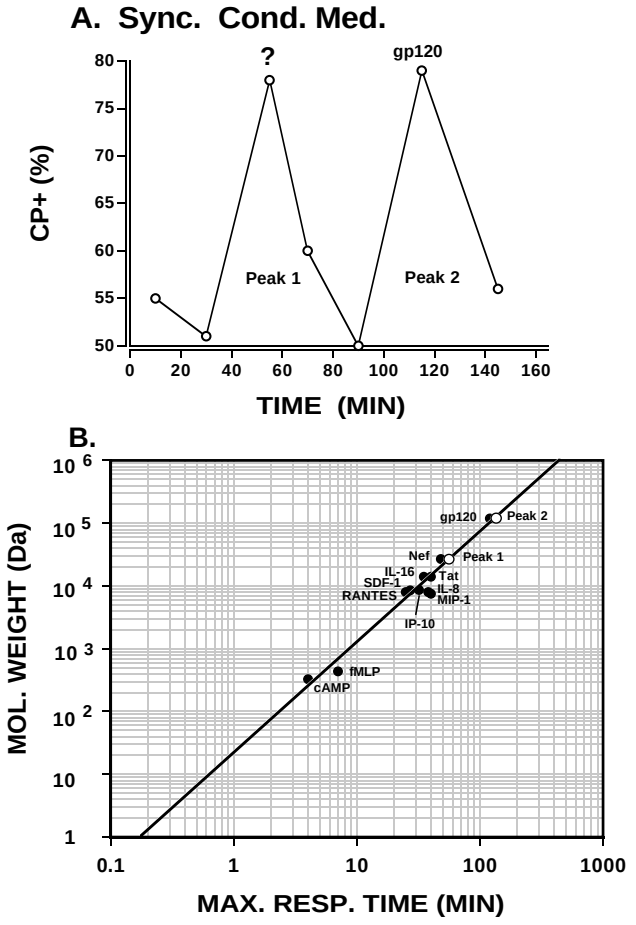

Fig. 1. Estimating the molecular masses of the two chemotactic activities in medium conditioned by HIV-induced T-cell syncytia. (A) The percentage of PBTHCs exhibiting a positive chemotactic response (CP+) plotted as a function of time for cells at the bridge of the single cell chemotaxis chamber in which HIV-induced syncytium-conditioned medium is placed in the source well and uninfected single cell-conditioned medium in the sink well. (B) Molecular mass of the gradient molecule as a function of the time it takes cells to exhibit a maximum response in the single cell chemotaxis chamber. Solutions of either a chemotactic or chemokinetic stimulant with known molecular masses were placed in the source well, known responsive cells were distributed at the bridge and buffer or medium lacking the stimulant was placed in the sink well of the single cell chemotaxis chamber described by Shutt et al. (1998). The time to achieve a maximum chemotactic or chemokinetic response was assessed by analyzing the average C.I. or CP+ in the former case and percentage chemokinetic stimulation in the latter case. Known molecules and responding cells included the following: cAMP (328 Da), *Dictyostelium* amoebae; fMLP (438 Da), human polymorphonuclear leukocytes; RANTES (8 kDa), peripheral blood T-helper cells (PBTHC); SDF-1 (8kDa), PBTHCs; IL-8 (8kDa), PBTHCs; IP-10 (8.7kDa), PBTHCs; MIP-1α (7.5kDa), PBTHCs; IL-16 (14 kDa), PBTHCs; Tat (14kDa), PBTHCs; Nef (27kDa), PBTHCs; gp120 (120 kDa), PBTHCs. The time it took to achieve a maximum cellular response to each stimulant was plotted as a filled circle. The times of maximum responses by PBTHCs to concentration gradients generated by HIV-induced syncytium-conditioned medium in the source well of the chemotaxis chamber (i.e. the two peaks in A) were plotted along the standard plot in B as unfilled circles. The estimated molecular masses of the two chemotactic activities responsible for peak 1 and peak 2 were approximately 30 kDa and 120 kDa, respectively. The identity of the 120 kDa chemoattractant was demonstrated in a prior study to be gp120 (Shutt et al., 1998).

Table 1. The chemotactic response of peripheral blood T-helper cells in the gradient chamber developed for the analysis of single cell behavior

Chamber‡		No. cells analyzed	Percentage CP+ (55 minutes)	C.I.
Sink	Source			
u.i. Cond. Med.	HIV-Sync. Cond. Med.	27	78	+0.31±0.49
sEBSS	sEBSS	37	54	+0.07±0.51
sEBSS	sEBSS + Rev	14	36	–0.06±0.46
sEBSS	sEBSS + p24	20	60	+0.07±0.43
sEBSS	sEBSS + Tat	20	40	+0.06±0.49
sEBSS	sEBSS + Nef	19	37	–0.06±0.46
sEBSS	sEBSS + Tat + Nef	40	80	+0.35±0.43
u.i. Cond. Med.	HIV-Sync. Cond. Med. + anti-Nef	16	50	+0.02±0.47
u.i. Cond. Med.	HIV-Sync. Cond. Med. + anti-Tat	15	40	+0.00±0.49
u.i. Cond. Med.	HIV-Sync. Cond. Med. + anti-Tat + anti-Nef	14	50	+0.08±0.47
sEBSS + 1/2 Nef	sEBSS + 1/2 Nef + Tat	23	52	+0.04±0.47
sEBSS + 1/2 Tat	sEBSS + 1/2 Tat + Nef	18	50	+0.01±0.46

*In all cases, the data presented are the pooled results of two or more independent experiments.

‡u.i. Cond. Med., supplemented RPMI medium conditioned by uninfected T-helper cells; HIV-Sync. Cond. Med., supplemented RPMI medium conditioned by HIV-induced syncytia; sEBSS, Earle's balanced salts solution supplemented with 2% normal human serum; anti-Tat, anti-Tat antibody; anti-Nef, anti-Nef antibody; 1/2 Nef, 1/2 Tat, half of the concentration of Nef or Tat that is used to generate a gradient when placed in the source alone.

concentration gradient of the syncytium-derived lower molecular mass chemotactic activity (Table 2).

Neither Tat, a transactivator of viral gene expression (Selby and Peterlin, 1990; Modesti et al., 1991), nor Nef, a negative regulator of viral replication (Garcia and Miller, 1991; Sawai et al., 1994; Aiken et al., 1994), functioned as a chemoattractant (Table 1). The average C.I. and CP+ of PBTHCs responding to a concentration gradient of Tat were +0.06±0.49 and 40%, respectively, and the average C.I. and CP+ for PBTHCs responding to a concentration gradient of Nef were –0.06±0.46 and 37%, respectively (Table 1). Again, the chemotactic behavior of PBTHCs in a gradient of Tat or Nef was similar to that in buffer (Table 1). However, both Tat and Nef functioned individually as chemokinetic stimulants. The percentage chemokinetic stimulation by Tat was 45% and that by Nef 27% (Table 2).

To illustrate the chemokinetic response of PBTHCs to a Tat or a Nef gradient in the absence of a chemotactic response, computer-generated perimeter tracks are presented for a field of PBTHCs in buffer alone, where there is neither chemotactic nor chemokinetic stimulation (Fig. 2A), in a concentration gradient of the lower molecular mass syncytium-derived chemoattractant, where there is both chemotactic and chemokinetic stimulation (Fig. 2B), in a concentration gradient of Tat, where there is chemokinetic but not chemotactic stimulation (Fig. 2C), and in a concentration gradient of Nef, where there is chemokinetic but not

chemotactic stimulation (Fig. 2D). In buffer alone, the perimeter tracks were short and the net directions of translocation relatively random (Fig. 2A). In a concentration gradient of the lower molecular mass syncytium-derived chemotactic activity, the perimeter tracks were, on average, longer than those in buffer, and the majority of cells exhibited net movement toward the source well (Fig. 2B). In a concentration gradient of Tat or Nef, the perimeter tracks were, on average, longer than those in buffer, but net directions were random (Fig. 2C and D, respectively). These behaviors are reflected in the measures of mean I.V., C.I. and CP+ derived for each representative field of cells and presented in the upper right-hand corner of each panel.

A combination of Tat and Nef functions as a chemoattractant

To test whether the chemokinetic stimulation by individual concentration gradients of Tat and Nef were additive or synergistic, PBTHC behavior was assayed in a chamber in which both Tat and Nef were added to the gradient source well. The concentration gradients of Tat and Nef together stimulated the average I.V. by 36%, approximately the same level of chemokinetic stimulation by either Tat or Nef alone (Table 2). However, in contrast to the random, non-directional behavior in either a Tat or Nef concentration gradient alone, cells in the combined gradient moved in a directed fashion towards the source well. The average C.I. of PBTHCs responding to the Tat

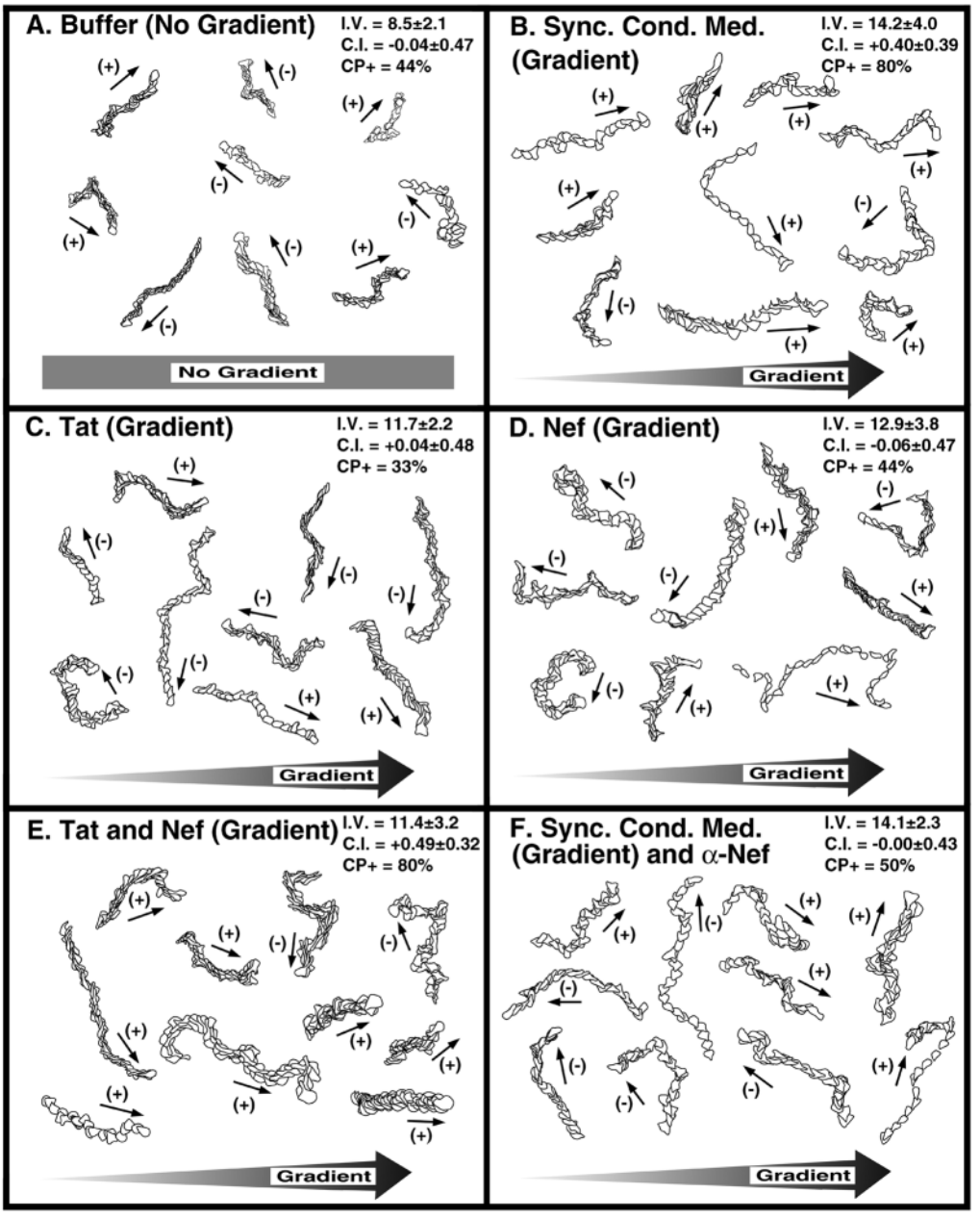

Fig. 2. Representative experiments depicting the behavior of individual cells at the bridge of the single cell chemotaxis chamber. Cell perimeters interpreted by the DIAS software program are drawn at 30 second intervals during the period of analysis to generate perimeter tracks. Responding PBTHCs were analyzed in buffer (A), during maximum response in a concentration gradient of the lower molecular mass syncytium-derived chemotactic activity (B), in a concentration gradient of Tat (C), in a concentration gradient of Nef (D), in simultaneous concentration gradients of Tat and Nef (E) and in a concentration gradient of the lower molecular mass syncytium-derived chemotactic activity in the presence of anti-Nef antibody (F). Cells were automatically analyzed with the computer-assisted DIAS software package (Soll, 1997; Soll and Voss, 1998). Cell behavior was analyzed in all cases during a 15 minute period between 47 and 63 minutes, which straddled the peak chemotactic and chemokinetic response of PBTHCs to the concentration gradient of the lower molecular mass attractant in syncytium-conditioned medium and the peak chemokinetic response of PBTHCs to a concentration gradient of either Tat or Nef. For each cell perimeter track, the net direction of translocation is noted by a small arrow, and a positive or negative chemotactic index is noted by a (+) or (-), respectively. The direction of the expected concentration gradient (the direction of increasing concentration) of each agent in B, C, D, E and F is noted by a large expanding arrow at the bottom of each panel. The average instantaneous velocity (I.V.), average chemotactic index (C.I.) and percentage of cells with a positive C.I. (CP+) for the cell population in each panel are presented in the upper right-hand corner of each panel. The width of each represented field in A-F is 550 μm. Sink and source solutions are described in Table 1.

Table 2. The chemokinetic response of peripheral blood T-helper cells in the gradient chamber developed for the analysis of single cell behavior*

Chamber‡		No. cells analyzed	Inst. vel. (μm/minute)	Percentage chemokinetic stimulation	Percentage motile cells
Sink	Source				
u.i. Cond. Med.	HIV-Sync. Cond. Med.	27	13.3±3.9	49	87
sEBSS	sEBSS	37	8.9±2.3	–	54
sEBSS	sEBSS + Rev	14	8.5±3.7	0	55
sEBSS	sEBSS + p24	20	6.5±2.4	0	52
sEBSS	sEBSS + Tat	20	12.9±3.7	45	61
sEBSS	sEBSS + Nef	19	11.3±2.8	27	65
sEBSS	sEBSS + Nef + Tat	40	12.1±4.1	36	86
u.i. Cond. Med.	HIV-Sync. Cond. Med. + anti-Nef	16	12.4±3.9	39	67
u.i. Cond. Med.	HIV-Sync. Cond. Med. + anti-Tat	20	14.8±3.2	66	84
u.i. Cond. Med.	HIV-Sync. Cond. Med. + anti-Nef + anti-Tat	18	14.1±4.0	58	79
sEBSS + 1/2 Nef	sEBSS + 1/2 Nef + Tat	23	12.7±3.3	43	75
sEBSS + 1/2 Tat	sEBSS + 1/2 Tat + Nef	18	12.6±2.5	42	80

*In all cases, the data presented are the pooled results of two more independent experiments.
‡Descriptions of the abbreviations are presented in Table 1.

plus Nef concentration gradients was +0.35±0.43, similar to the chemotactic index of +0.31±0.49 in a concentration gradient of the lower molecular mass syncytium-derived chemotactic activity, but in marked contrast to the average C.I. in either buffer (+0.07±0.51), in a concentration gradient of Tat alone (+0.06±0.49) or in a concentration gradient of Nef alone (–0.06±0.46; Table 1). The CP+ of PBTHCs responding to the gradients of Tat plus Nef was 80%, which was again similar to the CP+ of 78% in a concentration gradient of the lower molecular mass syncytium-derived chemotactic activity, but in marked contrast to the CP+ in buffer (54%), in a concentration gradient of Tat alone (40%) or in a concentration gradient of Nef alone (37%; Table 1). In Fig. 2E, perimeter tracks are presented for PBTHCs in the concentration gradients generated when both Tat and Nef are placed in the source well of the chamber. Note that the perimeter tracks are relatively long (chemokinesis) and that the majority of cells exhibit net movement toward the source of the gradient (chemotaxis). Chemokinetic and chemotactic stimulation are reflected in the measures of mean I.V., C.I. and CP+ derived for cells in the field and presented in the upper right-hand corner of the panel.

Nef and Tat together comprise the lower molecular mass chemoattractant activity released by HIV-induced T-cell syncytia

To test whether Tat and Nef together comprise the lower molecular mass chemotactic activity released by HIV-induced syncytia (Shutt et al., 1998), neutralization experiments were performed in which either anti-Tat or anti-Nef antibodies were added to both the sink and source solutions of a chamber with HIV-induced syncytium-conditioned medium in the source. PBTHC behavior was monitored for 65 minutes, a period that encompasses the response peak to the lower molecular mass activity (55 minutes, Fig. 1A). In buffer alone, the CP+ of

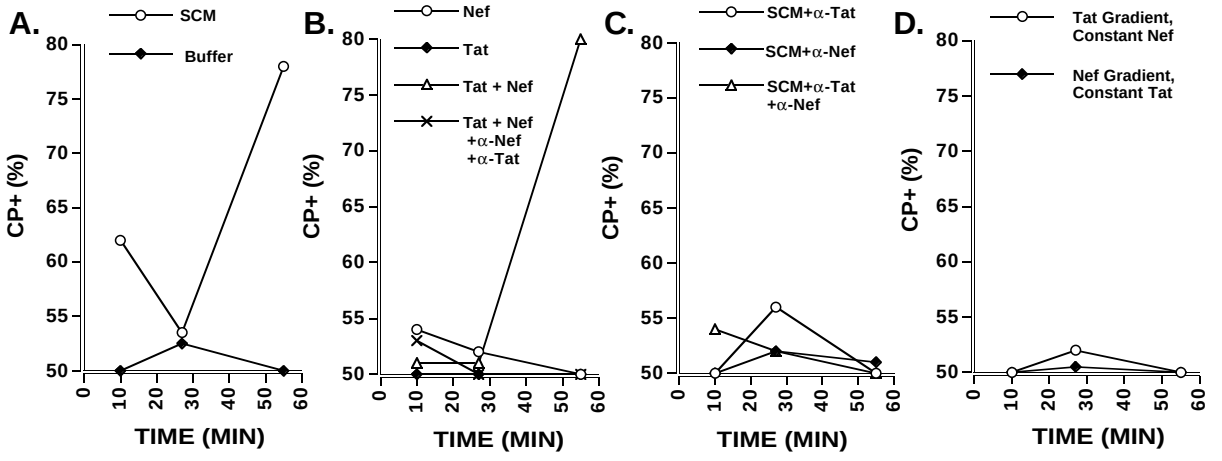

Fig. 3. Inhibition of chemotaxis in concentration gradients of the lower molecular mass syncytium-derived chemotactic activity by the addition of antibodies to Tat or Nef. The percentage of cells with a positive C.I. (CP+) was monitored over a 65 minute period for PBTHCs under the following conditions: (A) in buffer or in a concentration gradient of the lower molecular mass syncytium-derived chemotactic activity (SCM); (B) in a concentration gradient of Nef alone (Nef), in a concentration gradient of Tat alone (Tat), in simultaneous concentration gradients of Tat and Nef (Tat + Nef), and in concentration gradients of both Tat and Nef containing both anti-Tat and anti-Nef antibodies; (C) in concentration gradients of the syncytium-derived lower molecular mass chemotactic activity (SCM) plus anti-Tat antibody (SCM + α-Tat), plus anti-Nef antibody (SCM + α-Nef) or plus anti-Tat and anti-Nef antibodies (SCM + α-Tat + α-Nef); and (D) in a concentration gradient of Tat in which Nef is constant, or in a concentration gradient of Nef in which Tat is constant. Sink and source solutions are described in Table 1.

PBTHCs remained close to 50% throughout the 65 minute period of analysis (Fig. 3A). In a concentration gradient of the lower molecular mass syncytium-derived chemotactic activity without antibody, the CP+ increased to 78% between 30 and 55 minutes (Fig. 3A). In concentration gradients of Nef alone or Tat alone, the CP+ remained close to 50% throughout the 65 minute period of analysis (Fig. 3B), and in a concentration gradient of Nef plus Tat, the CP+ increased to 80% between 30 and 55 minutes (Fig. 3B). In a concentration gradient of the lower molecular mass syncytium-derived chemotactic activity in the presence of either anti-Nef or anti-Tat antibodies, or in the presence of both antibodies, the CP+ remained close to 50% (Fig. 3C). Therefore, anti-Tat antibody, anti-Nef antibody, or both, when incubated with syncytium-conditioned medium neutralized the lower molecular mass chemotactic activity in HIV-induced syncytium-conditioned medium. These results demonstrate that both Tat and Nef are essential components of the lower molecular mass chemoattractant released by HIV-induced syncytia.

The chemotactic response of T-helper cells to Tat and Nef requires parallel gradients

Although the preceding results demonstrate that both Tat and Nef are required for PBTHC chemotaxis, they do not demonstrate that both molecules must establish parallel gradients when functioning together as a chemoattractant. It is possible that either Tat alone or Nef alone must generate a gradient, while the other of the pair must simply be present at a constant concentration in order to elicit PBTHC chemotaxis. To test between these alternatives, experiments were performed in which one agent was placed in the source well to form a concentration gradient while the other agent was placed in both the source and sink wells to generate a constant concentration across the bridge of the chamber. Neither a Tat gradient with constant Nef, nor a Nef gradient with constant Tat elicited a chemotactic response. In each case, the C.I. was close to zero and the CP+ was close to 50% (Table 1; Fig. 3D). In both cases, however, the level of chemokinetic stimulation was similar to that in concentration gradients of each of the agents alone (Table 2). These results demonstrate that Tat and Nef must both establish gradients in order to elicit chemotaxis.

Tat and Nef do not function through the CD4 receptor

It was previously demonstrated that the addition of anti-CD4 antibody to the source and sink wells of a chemotaxis chamber with syncytium-conditioned medium in the source well blocked chemotaxis to the higher molecular mass chemoattractant (Shutt et al., 1998). However, neither anti-CD4 antibody nor recombinant soluble CD4 blocked the lower molecular mass chemoattractant (Fig. 4), demonstrating that the CD4 receptor does not represent the chemotactic receptor for either Tat or Nef.

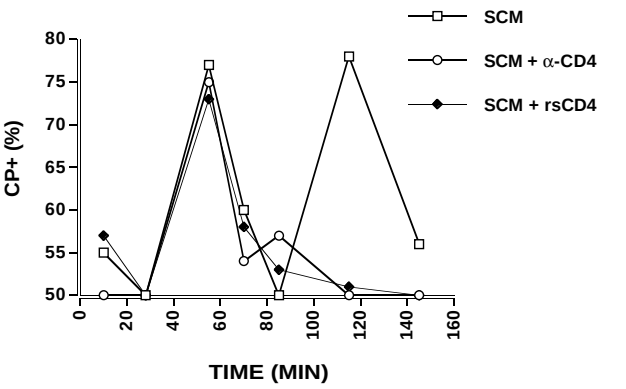

Fig. 4. Neither anti CD4-antibody nor recombinant soluble CD4 blocks the lower molecular mass chemoattractant in syncytium-conditioned medium. The percentage of PBTHCs with a positive C.I. (CP+) was monitored over time in a concentration gradient generated by the lower molecular mass chemoattractant in syncytium-conditioned medium (SCM), in syncytium-conditioned medium containing 4 μg per ml anti-CD4 antibody (SCM + α-CD4) and in syncytium-conditioned medium containing 2 μg per ml recombinant soluble CD4 (SCM + rsCD4). In the inhibition experiments, the blocking agent was also placed in the sink well containing uninfected T-cell conditioned medium at the indicated concentration.

DISCUSSION

HIV-induced T-cell syncytia have the remarkable capacity to reorganize their cytoskeleton and internal architecture in order to mimic the polarity and cellular morphology of single cells (Sylwester et al., 1993, 1995; Shutt et al., 1995b; Soll and Kennedy, 1994; Soll, 1997). These syncytia are motile, translocating along a substratum through the extension of a giant anterior pseudopod (Sylwester et al., 1993, 1995; Shutt et al., 1995b). Because of their capacity to extend and retract pseudopods, these syncytia are capable of disrupting collagen gels (Sylwester et al., 1998), of generating large holes in endothelium (Sylwester et al., 1998) and of phagocytosing single, uninfected T-cells (Murphy et al., 1995) in vitro. In videorecorded fields of single cells and syncytia in the act of fusion, it was apparent in some cases that single cells and small syncytia moved in a persistent and directed fashion towards large syncytia, suggesting that the latter released a T-helper cell chemoattractant (A. W. Sylwester and D. R. Soll, unpublished observations; Shutt et al., 1998).

To test this possibility, the motile behavior of T-helper cells was analyzed in gradients generated from medium conditioned by HIV-induced T cell syncytia. The microfilter assay, first introduced by Boyden (1962), was not used because of the inherent ambiguity it presents in distinguishing between chemotaxis and chemokinesis (Zigmond and Hirsch, 1973; Zigmond, 1978; Wilkinson, 1988; Rhodes, 1982). Instead, a gradient chamber was developed for continuously observing and recording the motile behavior of individual T-helper cells as steep molecular gradients formed and then flattened across the bridge of the chamber (Shutt et al., 1998). Computer-assisted motion analysis of each cell in the responding cell population then provided independent measures of increased cell velocity and directed movement towards the source of the gradient that discriminated between chemokinesis and chemotaxis, respectively. Using this chamber, we demonstrated that syncytia indeed release chemotactic activity, and by measuring the time it took to achieve maximum cell responses, we identified two chemotactic activities, one with an estimated molecular mass of approximately 30 kDa and one with an estimated molecular mass of approximately 120 kDa (Shutt et

al., 1998). Using antibody to gp120, we were able to neutralize the higher molecular mass chemotactic activity, and concluded that it was, in fact, the virally encoded glycoprotein gp120 (Shutt et al., 1998). This interpretation was reinforced by demonstrating that purified gp120 functioned as a bona fide chemoattractant for T-helper cells in the single cell chemotaxis chamber, and that antibody against the gp120 receptor CD4 blocked the higher molecular mass chemotactic activity (Shutt et al., 1998).

To test whether a low molecular mass protein encoded by HIV represented the lower molecular mass chemotactic activity released by HIV-induced syncytia, we first tested whether individual concentration gradients of Rev, p24, Tat or Nef stimulated single cell chemotaxis. Concentration gradients of the first two proteins, Rev and p24, were neither chemotactic nor chemokinetic stimulants of PBTHCs. Concentration gradients of neither Tat nor Nef stimulated chemotaxis of PBTHCs, but each elicited a strong chemokinetic response. To test whether the chemokinetic stimulation elicited by Tat alone and by Nef alone were additive, they were added together to the source well of the chemotaxis chamber and cell behavior monitored over time. To our surprise, we found that PBTHCs chemotaxed in response to a concentration gradient of this combination. The time necessary for T-helper cells to achieve a maximum chemotactic response was 55 minutes, approximately the same amount of time it took to achieve a maximum chemotactic response to a concentration gradient of the lower molecular mass chemotactic activity released by HIV-induced syncytia. This corresponds to a chemoattractant with an estimated molecular mass of approximately 20 to 30 kDa, using the standards curve in Fig. 1B. If Tat and Nef had combined in the source well to generate a concentration gradient as a heterodimer, we would have expected the peak of chemokinetic and chemotactic stimulation to have been approximately 75 minutes, based on their combined molecular mass of 41 kDa again using the standards curve in Fig. 1B. Since the peak response occurred at 55 minutes, this possibility seems unlikely. The data are more consistent with independent gradients of Tat and Nef. The molecular masses of Tat and Nef are 14 and 27 kDa, respectively. If chemotaxis requires independent gradients of both Nef and Tat, then the rate limiting gradient will be that of Nef, the highest molecular mass molecule and, therefore, the slowest gradient to form. The estimated time for the formation of a Nef gradient is approximately 55 to 60 minutes, the maximum response time for the low molecular mass chemoattractant.

Previous reports have suggested that Tat and Nef can affect the behavior of some cell types. Nef expression has been demonstrated to impair intracellular pathways involved in growth factor stimulation in HIV-infected astrocytes (Romero et al., 1998) and to affect selectively PI 3-kinase signaling pathways (Graziani et al., 1996). Although cell motility has not previously been demonstrated to be affected by the addition of extracellular Nef, an indirect effect on motility could be the result of growth factor stimulation and an alteration in PI 3-kinase signaling. There have been several reports demonstrating that extracellular Tat affects both the polarity and behavior of cells. Orsini et al. (1996) demonstrated that addition of Tat to a culture of rat cerebellar neurons caused them to aggregate and form spoked neurites. This report suggested that Tat affected adhesion and cellular morphology in a fashion similar to extracellular matrix proteins. Lafrenie et al. (1996a) demonstrated that the addition of Tat to cultures of primary monocytes increased their adhesion to endothelial monolayers, and concluded that Tat activated monocytes leading to extravasation through endothelium. In addition, Lafrenie et al. (1996b), Mitola et al. (1997), Benelli et al. (1997) and Albini et al. (1998) demonstrated that Tat stimulated migration of monocytes or dendritic cells across a membrane in Boyden chamber assays in a concentration-dependent fashion. Although these studies suggested that Tat stimulated chemotaxis, we contend that the microfilter assay does not definitively distinguish between a true chemotactic versus chemokinetic effect (Zigmond and Hirsch, 1973; Zigmond, 1978; Wilkinson, 1988; Shutt et al., 1998). Indeed, Zigmond (1978) noted that the results obtained from Boyden chamber measurements cannot discriminate between an increase in the proportion of responding cells, changes in the accuracy of orientation, changes in the frequency or the magnitude of turns, and changes in the rate of directed translocation, primarily because one cannot monitor single cell behavior with time. Rhodes (1982) has argued that even the checkerboard correction (Zigmond and Hirsch, 1973) may be inadequate for discriminating between chemotaxis and chemokinesis because it assumes that the behavior of cells crawling in the middle of a concentration gradient represents the average of the behaviors in non-gradient concentrations equivalent to the beginning and ending concentrations under gradient conditions. It has been demonstrated that animal cells respond in significantly different fashions depending upon whether the concentration of an attractant is increasing or decreasing with time (Zigmond and Sullivan, 1979; Varnum et al., 1985; Varnum-Finney et al., 1987; Wessels et al., 1992), reinforcing the reservations articulated by Rhodes (1982). Based upon our observations using a single cell chemotaxis chamber, in which the behavior of every cell in a test population is continuously assessed for both chemokinetic and chemotactic effects during the development and subsequent flatlining of steep gradients, we suggest that the chemotactic responses to Tat alone interpreted in previous studies using Boyden chamber-type devices may in fact have been chemokinetic responses. Alternatively, monocytes and dendritic cells, the cell types used in previous Boyden chamber experiments (Lafrenie et al., 1996b; Mitola et al., 1997; Benelli et al., 1998), may be truly capable of assessing a concentration gradient of Tat alone, unlike the PBTHCs analyzed in the present study. To resolve this question, experiments are now in progress in which the behaviors of individual dendritic cells and monocytes are being analyzed in gradients of Tat generated in the single cell chemotaxis chamber.

Having demonstrated that Tat and Nef in combination function as a PBTHC chemoattractant, we next tested whether they comprised the lower molecular mass chemotactic activity released by HIV-induced syncytia using antibodies to the two proteins in neutralization experiments. Antibody to either Tat alone or Nef alone neutralized the lower molecular mass chemotactic activity of syncytium-conditioned medium, demonstrating that a combination of the two proteins represents the lower molecular mass chemoattractant. As expected, a combination of anti-Tat and anti-Nef antibodies also neutralized chemotaxis, but did not block chemokinetic stimulation by syncytium-conditioned medium (Table 2). This

observation suggests that HIV-induced syncytia release other chemokinetic stimulants in addition to Tat and Nef.

The chemotactic activity of Tat plus Nef may either rely on the genesis of two parallel gradients, or on the genesis of a single gradient, with the requirement that the other agent simply be present. By generating either a Tat or a Nef concentration gradient in a constant concentration of the other, we have demonstrated that gradients of both molecules must form in order to elicit T-helper cell chemotaxis. Foxman et al. (1997) have presented evidence that neutrophils have the potential for navigating in sequence from one chemotactic gradient to another. To our knowledge, however, our results are the first to demonstrate a chemoattractant composed of two simultaneous molecular gradients using a method that discriminates unambiguously between chemotaxis and chemokinesis.

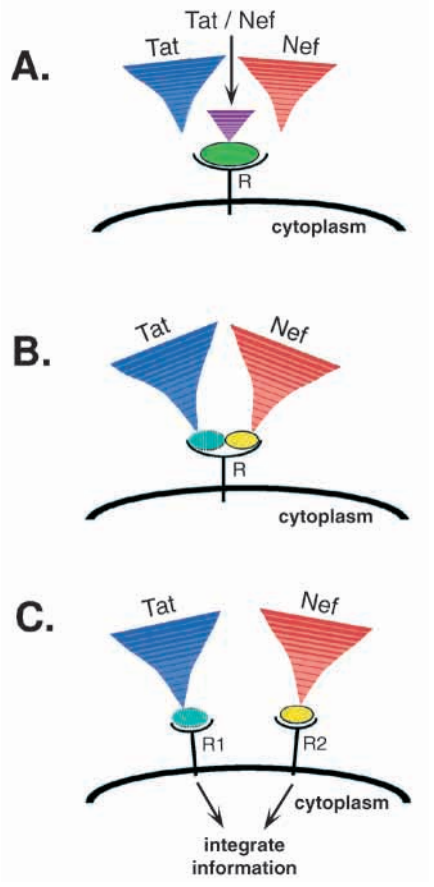

Fig. 5. Models for Tat plus Nef-stimulated chemotaxis of PBTHCs. In the three models, Tat, Nef or Tat/Nef gradients are cartooned as triangles with bands increasing in thickness to indicate the direction of increasing concentration towards the source. The receptors are labeled R. Model A: Tat and Nef form independent gradients, but a Tat/Nef complex occupies a single chemotactic receptor binding site. The Tat/Nef complex has a low binding constant. Therefore, the rate at which a Tat/Nef gradient forms at the cell body is dependent on the diffusion constants, and therefore molecular masses, of the individual Tat and Nef molecules, not the diffusion constant, and therefore molecular mass, of the Tat/Nef complex. This model has been shown to be inconsistent with our data (see Results). Model B: Tat and Nef form independent gradients and each independently interacts with two separate domains of the same receptor. Model C: Tat and Nef form independent gradients and each independently interact with different receptors, R1 and R2. In this model, the information resulting from receptor occupancy must be integrated in order for a cell to respond chemotactically.

There are several ways that cells may assess the directions of the two gradients and respond with directed motility. First, Tat and Nef may combine and as a complex occupy a single chemotactic receptor in a concentration-dependent fashion (Fig. 5A). We have demonstrated that the chemotactic response time in parallel gradients is consistent with the diffusion rates of the individual Tat and Nef molecules, not with the diffusion rate of a Nef/Tat complex formed in the source well. However, if the binding constant of Tat to Nef is low, the rate of gradient formation of the Tat/Nef complex at the cell body would be dependent upon the rates of gradient formation by the individual molecules, as diagrammed in Fig. 5A. This model seems unlikely since gradients of a Tat/Nef complex may also form at the cell body under conditions in which one molecule generated a gradient and the other remained constant. Since no chemotaxis was observed under the latter test conditions, the model in Fig. 5A may not be applicable. Second, Tat and Nef may each function independently in a concentration-dependent manner on the same chemotactic receptor (Fig. 5B). In this case, two parallel gradients would be necessary, and the receptor would be required to possess separate binding sites for Tat and Nef (Fig. 5B). Finally, Tat and Nef may each function in a concentration-dependent fashion on independent chemotactic receptors. In this latter model, cells would have to integrate the information of occupancy at the two receptors in order to assess direction (Fig. 5C). The correct model will emerge when the receptor, or receptors, for the two molecules are elucidated. Here, we have eliminated CD4, the chemotactic receptor for gp120 (Shutt et al., 1998), as a chemotactic receptor for either Tat or Nef.

The release of T-helper cell chemoattractants from infected tissue can have immediate pathogenic consequences. Since lymph nodes and other lymphoid tissues can harbor high densities of virally infected cells, they may function as the source of concentration gradients of Tat, Nef and gp120, inducing T-helper cells to extravasate from blood vessels into the infected tissue, where they would have a higher probability of infection or bystander death (Finkel et al., 1995). In fact, lymphadenopathy, the enlargement of lymph nodes, often occurs at the time that CD4-positive T-helper cells decrease in concentration in the peripheral blood and increase in concentration in the lymph nodes of HIV-infected individuals (Janossy et al., 1985; Rosenberg et al., 1993). Specific receptors have recently been implicated in this homing process (Wang et al., 1999). Blocking chemotaxis to the HIV-encoded T-helper cell chemoattractants may, therefore, represent a therapeutic strategy for slowing HIV-related disease progression.

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