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T cell syncytia induced by HIV release

T cell chemoattractants: demonstration with a newly developed single cell chemotaxis chamber

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SUMMARY

A chemotaxis chamber has been developed to analyze both the velocity and the directionality of individual T cells in gradients of high molecular mass molecules over long periods of time. Employing this chamber, it is demonstrated that syncytia induced by HIV in SUP-T1 cell cultures release two T cell chemoattractants with approximate molecular masses of 30 and 120 kDa. Neither uninfected single cells nor polyethylene glycol-induced syncytia release detectable chemoattractant, suggesting that these chemoattractants are linked to HIV infection. Soluble gp120 functions as a T cell chemoattractant and the addition of anti-gp120 antibody to syncytium-conditioned medium blocks the high molecular mass chemoattractant activity but not the low molecular mass activity. The addition of anti-CD4 antibody to syncytium-conditioned medium also blocks the high molecular mass chemoattractant activity but not the low molecular mass activity. These results demonstrate that HIV-induced T cell syncytia release a low and a high molecular mass T cell chemoattractant, and suggest that the high molecular mass factor is gp120 and that it functions through the CD4 receptor.

Key words: HIV-induced syncytium, T cell chemotaxis, gp120

INTRODUCTION

When T cells are productively infected with the human immunodeficiency virus (HIV), they express the virally encoded protein gp120 and gp41 on their surface (Kowolski et al., 1987; McDougal et al., 1986). Surface gp120 in turn can interact with the CD4 receptor of an uninfected cell to initiate cell fusion and the genesis of a multinucleated syncytium (Lifson et al., 1986a,b). Through subsequent gp120/gp41-mediated fusions, syncytia can grow to large sizes, and in cultures of an infected T cell line, fusion can account for the majority of cell death (Sylwester et al., 1997). Although HIV-induced syncytia are often considered to be disorganized, short lived fusion products in the throes of death, recent results have demonstrated that these syncytia have the remarkable capacity to mimic the subcellular organization of single cells, develop cellular polarity, extend a single, giant pseudopod and crawl with the same behavioral characteristics as single T cells (Sylwester et al., 1993, 1995; Shutt et al., 1995a; Soll, 1997). HIV-induced syncytia also acquire the capacity to phagocytose uninfected T cells by engulfing them through extension of their giant pseudopodia (Murphy et al., 1995). Syncytia formed in HIV-infected cell cultures lyse on average after 48 hours, but stimulate new syncytium formation in their general vicinity (Sylwester et al., 1997). Eventually the majority of cells enter syncytia in an HIV-infected SUP-T1 cell culture through fusion (Sylwester et al., 1997), and there are indications that single cells first interact with a syncytium prior to fusion through the extension of a pseudopod (Sylwester et al., 1993).

In the computer-assisted studies in which the motile behaviors of syncytia were assessed (Sylwester et al., 1993, 1995, 1997; Shutt et al., 1995a), infected T cell populations were continuously videorecorded at either low or high magnification for periods ranging from 5 minutes to 7 days. In a number of videoanalyzed culture fields, there were indications that single cells sometimes moved in a directed fashion towards syncytia, over distances too great to be accounted for by filopodial or pseudopodial contact (A. Sylwester, D. Wessels, R. Kennedy, D. Shutt, and D. R. Soll, unpublished observations), suggesting that syncytia may release a T cell chemoattractant. To test whether syncytia release a T cell chemoattractant, we chose to use a method for assessing individual cell behavior in a chemical gradient (Zigmond, 1977, 1978; Varnum-Finney et al., 1987) rather than a micropore filter assay, since the latter method does not always effectively discriminate between chemotactic and

chemokinetic stimulation (Zigmond, 1978; Zigmond and Hirsch, 1973; Wilkinson 1988; Rhodes, 1982; also, see Discussion). A gradient chamber was designed which provides a conditioned surface to maximize single T cell motility and which is enclosed to facilitate the analysis of single cell chemotaxis toward high molecular mass attractants which require long time periods for the establishment of gradients. Using this newly developed single cell chemotaxis chamber, we provide evidence that HIV-induced T cell syncytia release two potent T cell chemoattractants with predicted molecular masses of approximately 30 and 120 kDa. The latter attractant has approximately the same molecular mass as the virally encoded gp120, which is demonstrated here to be a potent single cell chemoattractant. The activity of the high molecular mass attractant but not that of the low molecular mass attractant is selectively blocked by anti-gp120 antibody and anti-CD4 antibody, suggesting that the high molecular mass attractant is gp120 and functions through the CD4 receptor. The low molecular mass attractant remains unidentified.

MATERIALS AND METHODS

Preparation and culture of T cells

Cells of the immortalized T cell line SUP-T1 (Smith et al., 1984) were maintained according to methods previously described (Sylwester et al., 1993, 1995, 1997; Shutt et al., 1995a). To obtain a highly enriched culture of CD4+ peripheral blood T cells, fresh blood (250 cm^2) was drawn from a healthy HIV-negative donor into heparinized syringes and 31 ml were overlaid on 15 ml of Ficoll-Hypaque solution in each of eight 50 ml conical tubes. The tubes were centrifuged for 40 minutes at 550 ***g***, and the band of mononuclear cells atop each cushion retrieved and pooled into a fresh 50 ml conical tube. The cells were pelleted at 1,000 ***g*** for 10 minutes and washed in Earle's balanced salts solution (EBSS). The final pellet was 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), 2 mM L-glutamine, 1× MEM non-essential amino acid solution, 1 mM Na-pyruvate, 10 mM Hepes, 100 units/ml penicillin and 100 μg/ml streptomycin sulfate ('supplemented RPMI medium'). The cell suspension was transferred to two T-75 culture flasks and incubated for 2 hours at 37°C in 5% CO_2. Non-adherent cells were transferred to a 50 ml conical tube, gently pelleted, resuspended in EBSS, and counted in a haemocytometer. 1.25×10^8 cells were passed through a Cellect human CD4+ T cell negative selection column (Biotex Laboratories Inc., Edmonton, Canada) according to the manufacturer's protocol. The flow-through cells were pelleted and resuspended at a final concentration of 1×10^6/ml in supplemented RPMI medium containing 20% FBS, 10 μg/ml phytohemagglutinin and 10 units/ml delectinated IL-2. This procedure was previously demonstrated to generate cell cultures containing greater than 90% CD4+ T cells (Shutt et al., 1995a), and for simplicity in the Results and Discussion, cells in this final preparation have been referred to as 'PBTCs'. Before use in chemotaxis experiments, PBTCs were grown in the latter stimulation medium for a minimum of 48 hours.

Dictyostelium discoideum, strain AX3, were stored, cultured and developed to the ripple stage to obtain chemotactically competent amoebae according to methods previously described in detail (Soll, 1987; Wessels et al., 1989). Human polymorphonuclear leukocytes were collected according to methods previously described (Murray et al., 1992).

Infection with HIV and collection of syncytium-conditioned medium

HIV-1LAI (previously referred to as HTLV-IIIB) was passed in cell cultures according to methods previously described (Sylwester et al., 1993, 1995, 1997; Shutt et al., 1995a). For infection, 10^7 SUP-T1 cells were pelleted, resuspended in 1 ml of infected culture supernatant containing 5,000 $TCID_{50}$ of HIV-1LAI and incubated for 2 hours. Parallel mock infections were performed by resuspending the cells in medium lacking virus. After incubation, cells were diluted with fresh supplemented RPMI medium. To obtain syncytium-conditioned supernatant, 72-120 hours post-infected cultures in Petri dishes were placed on the stage of an inverted microscope positioned in a biosafety cabinet, and approximately 800 individual syncytia were individually transferred with a micropipette to a 1.5 ml screw cap tube containing 0.5 ml of supplemented RPMI medium. Syncytia were incubated for 6 hours at 37°C in a 5% CO_2-air mixture. Syncytia were then gently pelleted, and the supernatant removed and stored at −20°C. For medium conditioned by syncytia induced by polyethylene glycol (PEG) (see next section of methods), the total volume of PEG-induced syncytia was estimated and converted to 'single cell volume equivalents' by methods previously described (Sylwester et al., 1997). A concentration of PEG induced syncytia or uninfected cells equivalent to the estimated total single cell volume of 800 HIV-induced syncytia were then incubated in fresh medium in the same manner as HIV-induced SUP-T1 syncytia to obtain the respective conditioned media.

Preparation of polyethylene glycol-induced syncytia

The method used was adapted from that of Vaughan et al. (1976). SUP-T1 cells were pelleted, resuspended in serum-free RPMI medium at a concentration of 1×10^7 cells per ml, and 1 ml aliquots added to each of a series of small vials in a 37°C waterbath. To each vial, 1 ml of a prewarmed (37°C) solution of serum-free RPMI medium containing 50% (w/v) polyethylene glycol 1500 (Sigma Chemical Co., St Louis, MO) was added, and the solution gently mixed over a 1 minute period. At 1 minute intervals, 1 ml aliquots of Hanks' buffered salts solution (HBSS) were added and the vials continuously mixed. After a 10 minute period, cells were pelleted, gently resuspended in 5 ml of HBSS and incubated at 37°C for 5 minutes. Cells were then pelleted, resuspended in 5 ml of supplemented RPMI medium and allowed to recover for 30 minutes at 37°C. The resulting syncytia, which averaged 20-30 single cell volume equivalents were then transferred and used to condition medium as described.

The chemotaxis assay

Cultures of SUP-T1 cells or PBTCs were grown to a density of 1×10^6 cell per ml, and 200 μl of the culture were pipetted onto the center of a 22×30 mm Thermanox® tissue culture-treated plastic coverslip (NUNC Inc., Naperville, IL) positioned in the middle of a plastic Petri dish. This culture was incubated for approximately 20 hours. Conditioning the surface of the coverslip in this manner increased the proportion of motile cells in subsequent assays approximately 10-fold (data not shown). Excess medium was gently removed from the coverslip, taking care not to disturb the attached cells. The coverslip was inverted and clamped onto the inverted gradient chamber diagrammed in Fig. 1. The chamber in turn was inverted. To generate a gradient, one well was arbitrarily designated the 'sink' and filled with buffer or medium lacking the test molecule, and the other well was designated the 'source' and filled with solution containing the test attractant. A glass coverslip was lowered over the wells and bridge, beginning at the sink to minimize contamination of the sink solution with the source solution. The chamber was positioned on the stage of a Zeiss Axiovert 100 microscope equipped with long distance objectives and condenser. A Plexiglas chamber, which enclosed the microscope stage and

gradient chamber, was perfused with a humidified 5% CO_2-air mixture. Temperature was maintained at 37°C with a custom-built thermostatically controlled stage heater. In the genesis of spatial gradients, the following concentration of test molecules were individually used in the source well of the chamber: 10^{-6} M cAMP (Sigma Chemical Co., St Louis, MO) for *Dictyostelium discoideum* (Varnum-Finney et al., 1987; Shutt et al., 1995b); 5×10^{-8} M fMLP (Sigma Chemical Co.) for human polymorphonuclear leukocytes (Zigmond, 1977); 1 μg per ml gp120 from HIV-1_{JR-FL} (a generous gift from Dr P. Maddon, Progenics Inc., Tarrytown, NY) for T cells (Kornfeld et al., 1988); 3×10^{-11}M IL-16 (a generous gift from Dr W. W. Cruikshank, Boston University) for T cells (Cruikshank et al., 1991); and 200 ng/ml RANTES (from Life Technologies, Gaithersburg, MD) for T cells (Schall et al., 1990). Anti-gp120 antibody (cat. no. 567) was obtained through the AIDS Research and Reference Reagent Program of NIH, donors Dr M. Page and Dr R. Thorpe, MRC AIDS Directed Programme, Great Britain. Anti-CD4 antibody Primo-1 has been described elsewhere (Attanasio et al., 1991).

Computer-assisted analysis of single cell behavior

Cells on the bridge of the spatial gradient chamber were continuously monitored with a CCD camera (Cohu, Inc., San Diego, CA) through a 10× objective. Cell images were directly digitized at 15 second time intervals using a frame-grabber (Data Translation, San Diego, CA) installed in a Macintosh 7100 Power PC computer (Apple Computer Inc., Cupertino, CA). Images were processed and cell perimeters automatically determined at each time point with DIAS software according to methods previously described in detail (Soll, 1995; Soll and Voss, 1997). The position of the cell centroid at each time point was computed as the center of mass of the β-spline replacement image (Soll, 1995). The instantaneous velocity (I.V.) of each cell was computed at each time point (f) by the central difference method (Maron, 1982) according to the formula:

$$\text{I.V.(f)} = \frac{d1 + d2}{2\Delta t} \quad ,$$

where d1 and d2 are the distances 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. The average instantaneous velocity of a cell was then calculated by taking the sum of all I.V. measurements and dividing by the total number of time intervals. The instantaneous velocity was used in assessing the chemokinetic stimulation by a test factor or known molecule.

Chemotactic index (C.I.) was computed as the net distance a cell moved in the direction of the attractant (source well in chamber, Fig. 1) divided by the total distance moved. A C.I. of +1.0 represents direct movement towards the source well, a C.I. of −1.0 represents direct movement away from the source well, and a C.I. of 0.0 represents no net movement towards or away from the source. Positive chemotactic indices for the cell types tested in the chamber originally pioneered by Zigmond and coworkers range between +0.15 and +0.70 (Zigmond, 1977; Varnum-Finney et al., 1987; Shutt et al., 1995b). CP+ represents the proportion of translocating cells in a population that exhibit a positive chemotactic index. A CP+ of 50% represents random movement by cells in an analyzed population, while a CP+ of 100% represents positive chemotactic indices for 100% of the cells. In all cases in which C.I., CP+, and I.V. were computed for a single time period in a gradient chamber, only cells which exhibited average instantaneous velocities above 3.0 μm per minute and which remained in the field of analysis for a minimum of 8 minutes were analyzed. The mean C.I., mean CP+ and mean instantaneous velocity were computed from the pooled data of two or more replicate experiments.

RESULTS

A chamber designed to test individual T cell chemotaxis in gradients of high molecular mass molecules

A gradient chamber, designed according to the specifications of Zigmond (1977) that proved to be highly successful in assessing the chemotaxis of individual cell in gradients of low molecular mass molecules (e.g. Varnum-Finney et al., 1987; Shutt et al., 1995b; Zigmond, 1978) was unsuccessful in assessing the chemotactic responsiveness of SUP-T1 cells to HIV-induced syncytium-conditioned medium for three reasons. First, the bridge of the gradient chamber separating the source and sink wells was composed of plexiglass and was a fixed component of the apparatus. Therefore, conditioning the surface with T cells overnight (O'Neill and Parrott, 1977) could be performed only once per chamber per experiment. More importantly, plexiglass was not easily conditioned, and conditioning was necessary for efficient T cell translocation. Second, when the coverslip of the chamber was used as an inverted surface for supporting cells over the bridge, cells fell from the inverted coverslip during the long incubation periods required to form gradients of high molecular mass molecules (Lauffenberger et al., 1988), even when the coverslip surface was conditioned. Third, because the wells of this chamber were open to air, evaporation disorganized gradients during the extended time periods necessary to establish gradients of high molecular mass molecules. A chamber was, therefore, designed which contained: (1) enclosed wells that remove the problem of evaporation; (2) a removable tissue culture plastic surface for supporting cells at the bridge of the chamber, allowing several to be individually conditioned in parallel prior to an experiment; and (3) a supporting surface that is right-side up to minimize the release of cells during long incubation times (Fig. 1).

To test the efficacy of this chamber (Fig. 1), two chemotactically responsive cell types that had previously been well characterized in gradient chambers, the soil amoeba *Dictyostelium discoideum* (Varnum-Finney et al., 1987; Shutt et al., 1995b; Wessels et al., 1988) and human polymorphonuclear leukocytes (Zigmond, 1977), were analyzed for their response to their respective chemoattractants, cAMP and fMLP. Instantaneous velocity and the two chemotactic parameters, the average chemotactic index (C.I.) and the percentage of cells exhibiting a positive chemotactic index (CP+), were computed according to the formulas and thresholds outlined in Materials and Methods. Individual *D. discoideum* amoebae exhibited random movement at the chamber bridge when buffer was placed in both wells, and this is demonstrated in Fig. 2A, in which the individual cell tracks and the net directonal vector of each cell is presented for a 10 minute period of analysis. To assess whether behavior was random, one well was arbitrarily designated the 'source', even though it contained no attractant, in order to compute the average C.I. and CP+. If cells moved randomly, the average C.I. would be close to 0.00 and the CP+ close to 50%. The average C.I. of cells in the example in Fig. 2A was −0.06 and the CP+ 50%, reflecting random movement. In marked contrast, when *D. discoideum* amoebae were placed at the bridge of a chamber in which buffer was placed in one

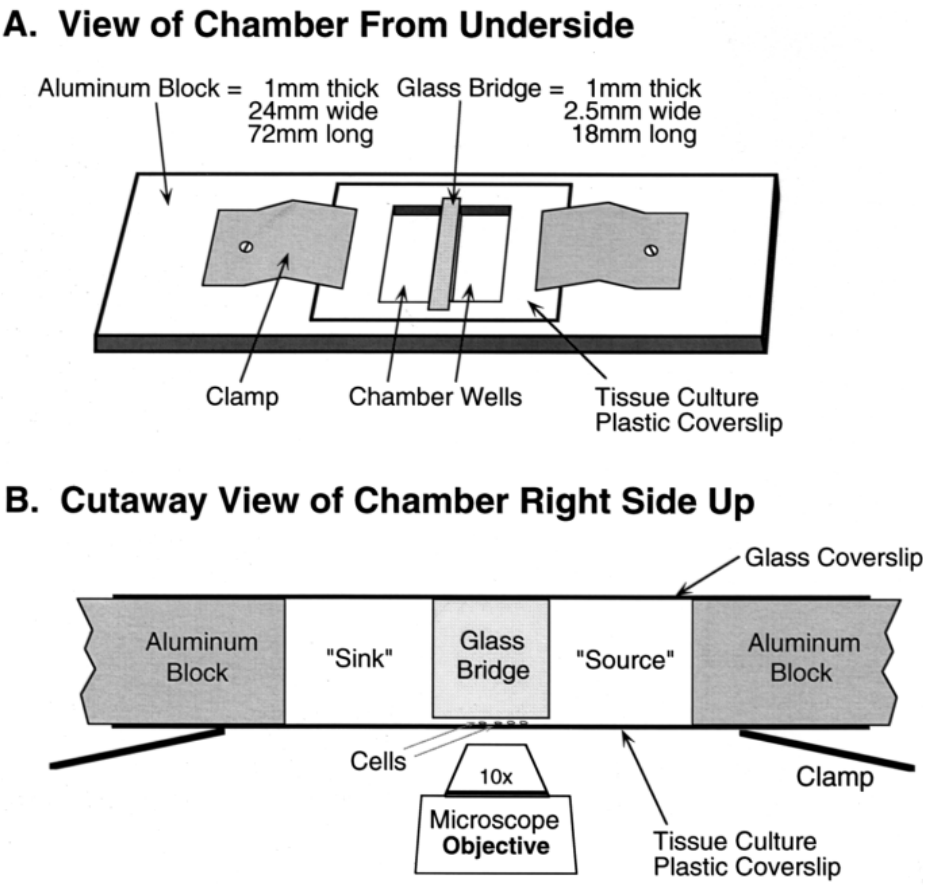

Fig. 1. A customized chamber for the analysis of single cells in gradients of chemoattractant. The important features of this chamber include an upright cell-supporting surface made of tissue culture plastic which is removable and can be conditioned with single cells overnight, and 'sink' and 'source' wells which are sealed from the air to inhibit evaporation during the long time periods necessary for generating gradients of high molecular mass molecules. (A) A view from the chamber underside. This view shows clamps which press the tissue culture plastic coverslip against the underside of the aluminum block. The glass bridge and chamber wells are visible through the coverslip. (B) Cutaway view of the chamber right side up. Note the clamps are now underneath the chamber and the cells are distributed on the plastic coverslip under the bridge between the source and sink wells.

well and buffer containing 10^{-6} M cAMP was placed in the other well, the amoebae exhibited directed movement towards the source of the gradient (Fig. 2B). In the example in Fig. 2B, amoebae exhibited an average C.I. of +0.63 and a CP+ of 90% upon establishment of a gradient of cAMP (i.e. after approximately 4 minutes of incubation). This C.I. is in the high range of values reported for this cell type (Varnum-Finney et al., 1987; Shutt et al., 1995b; Varnum and Soll, 1984; Chen et al., 1995). Peak chemotactic efficiency occurred 4 minutes after initiation of the gradient (data not shown), which is consistent with the time it would take to generate a gradient based on the specifications of the chamber, the molecular mass of cAMP and Einstein's diffusion equation (Lauffenberger et al., 1988). Individual human polymorphonuclear leukocytes (PMNs) also exhibited random behavior when buffer was placed in both wells of the chamber (Fig. 2C) and directed movement towards the source of a gradient of fMLP (Fig. 2D). In the former case PMNs exhibited an average C.I. of –0.05 and a CP+ of 40%, and in the latter case an average C.I. of +0.50 and a CP+ of 100%. This C.I. is in the upper range of C.I.s reported for PMNs (Zigmond, 1977, 1978; Lackie and Burns, 1983). Peak chemotactic efficiency occurred in the chamber at 7 minutes after initiation of the gradient (data not shown), which again is consistent with the molecular mass of fMLP (Lauffenberger et al., 1988). The results with both *D. discoideum* amoebae and human PMNs demonstrate the efficacy of the newly developed chamber (Fig. 1) in analyzing chemotaxis at the single cell level in gradients of low molecular mass molecules.

HIV-induced SUP-T1 syncytia release two T cell chemoattractants

To test whether HIV-induced syncytia of the SUP-T1 cell line release a chemoattractant, SUP-T1 cells were infected with HIV-1LAI. After 72 to 120 hours, significant numbers of large syncytia had accumulated in the culture (Sylwester et al., 1997). Approximately 800 syncytia with volumes averaging 150 single cell volume-equivalents were removed from the culture with a micropipette and incubated for 6 hours in 0.5 ml of supplemented RPMI medium. The syncytia were then gently pelleted and the supernatant added to the 'source' well of the gradient chamber (Fig. 1). Supplemented RPMI medium previously conditioned with uninfected SUP-T1 cells was added to the 'sink' well of the chamber (Fig. 1). Different fields of SUP-T1 or PBTC cells at the chamber bridge (Fig. 1) were videorecorded for 10 to 30 minute segments in sequence during a continuous period of 120 to 150 minutes. The behavior of individual cells in each sequence was then analyzed with DIAS software (Soll, 1995; Soll and Voss, 1997). Positive chemotactic behavior, in this case assessed as a CP+ above 60%, peaked twice, at 60 minutes and at 120 minutes (Fig. 3A), suggesting that medium conditioned by HIV-induced syncytia generated two chemotactic gradients, one by a low molecular mass attractant, which peaked at 60 minutes, and one by a high molecular mass attractant, which peaked at 120 minutes. To estimate the molecular masses of the two chemoattractants in HIV-induced syncytium-conditioned medium, a curve was generated for known chemotactic and chemokinetic stimulants in which the log of molecular mass was plotted as a function

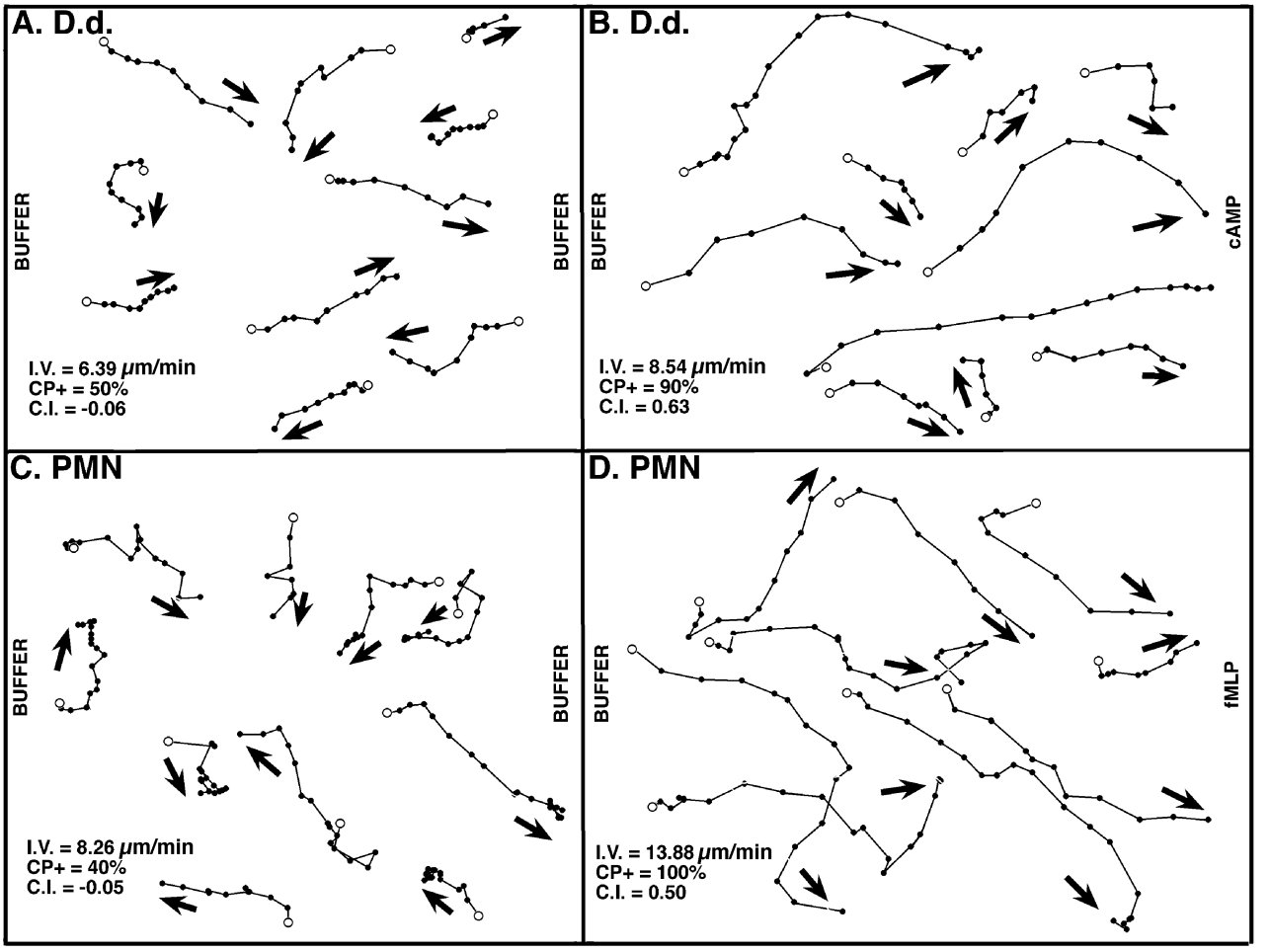

Fig. 2. Chemotaxis of *Dictyostelium discoideum* (D.d.) amoebae in a gradient of cAMP and human polymorphonuclear leukocytes (PMN) in a gradient of fMLP established in the customized chamber described in Fig. 1. The computed centroid of each cell is plotted at 1 minute intervals over periods of continuous translocation. The centroids are connected to form translocation tracks. The centroid at the beginning of each track is noted by an unfilled circle. The net direction of translocation is noted by an arrow for each track. Over 70% of the cells in each field were motile, and only motile cells are shown. (A) Random behavior of D.d. amoebae in a chamber in which buffer is placed in both wells. (B) Directed movements of D.d. amoebae toward the source of a gradient of cAMP in a chamber in which buffer is placed in a 'sink' well and 10^{-6} M cAMP in a 'source' well. (C) Random behavior of human PMNs in a chamber in which buffer is placed in both wells. (D) Directed movement of PMNs in a chamber in which buffer is placed in a sink well and 5×10^{-8} M fMLP in a source well. I.V., instantaneous velocity; CP+, percentage motile cells exhibiting a net positive chemotactic response; C.I., average chemotactic index.

of the log of maximum response time in the chamber (Fig. 4). Test molecules for responsive cells included the chemoattractant cAMP for *D. discoideum* amoebae, the chemoattractant fMLP for human PMNs, and RANTES, IL-16, and gp120, which we found functioned as chemokinetic agents for T cells (D. Shutt and D. R. Soll, in preparation; also, Table 2). The data points fell in a straight line. Based on the times of the two CP+ peaks P1 and P2, representing the low and the high molecular mass attractants, respectively, the extimated molecular masses were estimated to be approximately 30 and 120 kDa, respectively (Fig. 4). The average C.I. of responding SUP-T1 cells at the low molecular mass peak was +0.23 and the CP+ 67%, and the average C.I. at the high molecular mass peak was +0.27 and the CP+ 80% (Table 1). A control was performed in which Earle's balanced salts solution (EBSS) was placed in both wells of the chamber. Through 130 minutes of analysis, there was no significant increase in CP+ (Fig. 2F). The average C.I. for SUP-T1 cells after 115 minutes in EBSS was −0.01 and the CP+ 45% (Table 1), demonstrating random movement.

The above analysis was repeated with uninfected peripheral blood T cells (PBTCs) rather than SUP-T1 cells as responders and the results were similar. In a chamber in which syncytium-conditioned medium was placed in the source well and unconditioned medium was placed in the sink well, the CP+ peaked at 55 and 120 minutes (Fig. 3A), again demonstrating two chemoattractants at approximately 30 and 120 kDa (Fig. 4). In the case of PBTCs, the average C.I. and CP+ for the first peak were +0.27 and 73%, respectively, and for the second peak +0.36 and 85%, respectively (Table 1). In a control experiment in which EBSS was placed in both wells of the chamber, the C.I. was +0.06 and CP+ 48% for PBTCs (Table 1), again demonstrating random movement.

Table 1. Chemotactic response of different T cells to known chemoattractants or to test solutions in the chemotactic chamber described in Fig. 1

Cell type	No. cells analyzed	Chamber*		Period of analysis (min)	Chemotactic index (C.I.)	Percentage positive (CP+)
		Sink	Source			
SUP-T1	18	u.i. Cond.med.	H-Sync.cond.med.†	50-70	+0.23	67%
	30	u.i. Cond.med.	H-Sync.cond.med.‡	110-130	+0.27	80%
	48	u.i. Cond.med.	P-Sync.cond.med.	110-130	+0.00	54%
	58	EBSS	gp120	110-130	+0.26	79%
	38	EBSS	EBSS	100-130	–0.01	45%
	19	EBSS	u.i. cond.med.	100-130	–0.07	53%
(CD4+)PBTC	19	u.i. Cond.med.	H-Sync.cond.med.†	45-65	+0.27	73%
	20	u.i. Cond.med.	H-Sync.cond.med.‡	110-130	+0.36	85%
	21	EBSS	gp120	115-135	+0.33	81%
	31	EBSS	EBSS	100-130	–0.03	48%
	25	EBSS	u.i. cond.med.	100-130	–0.03	44%
	14	u.i. Cond.med.	H-Sync.cond.med.+anti-gp120†,§	45-65	+0.16	67%
	12	u.i. Cond.med.	H-Sync.cond.med.+anti-gp120‡,§	110-130	+0.01	57%
	33	u.i. Cond.med. +anti-CD4	H-Sync.cond.med+anti-CD4†,¶	45-65	+0.17	76%
	53	u.i. Cond.med. +anti-CD4	H-Sync.cond.med+ anti-CD4†,¶	110-130	+0.00	53%
	26	u.i. Cond.med. +anti-CD4	u.i. Cond.med+anti-CD4†,¶	45-65	+0.03	54%
	21	u.i. Cond.med. +anti-CD4	u.i. Cond.med+anti-CD4†,¶	110-130	–0.07	43%

*u.i. Cond.med., supplemented RPMI conditioned by uninfected SUP-T1 cells; H-Sync.cond.med., supplemented RPMI conditioned by HIV-induced SUP-T1 syncytia; P-Sync.cond.med., supplemented RPMI conditioned by polyethylene glycol-induced syncytia; EBSS, Earle's balanced salts solution; Sync.cond.med.+anti-gp120, supplemented RPMI conditioned by HIV-induced SUP-T1 syncytia containing 1:20 polyclonal anti-gp120 antibody; u.i. Cond.med.+antiCD4, supplemented RPMI conditioned by HIV-induced SUP-TI syncytia containing 4 mg/ml monoclonal anti-CD4.

†Early peak of chemotaxis (resolved from time plots like the ones in Fig. 3).

‡Later peak of chemotaxis (resolved from time plots like the ones in Fig. 3).

§HIV-induced syncytium-conditioned medium was incubated with anti-gp120 antibody for 15 minutes prior to placing the solution in the source well of the chamber.

¶Responding cells and both sink and source solutions were treated with 4 mg/ml anti-CD4 antibody for 5 minutes prior to analysis.

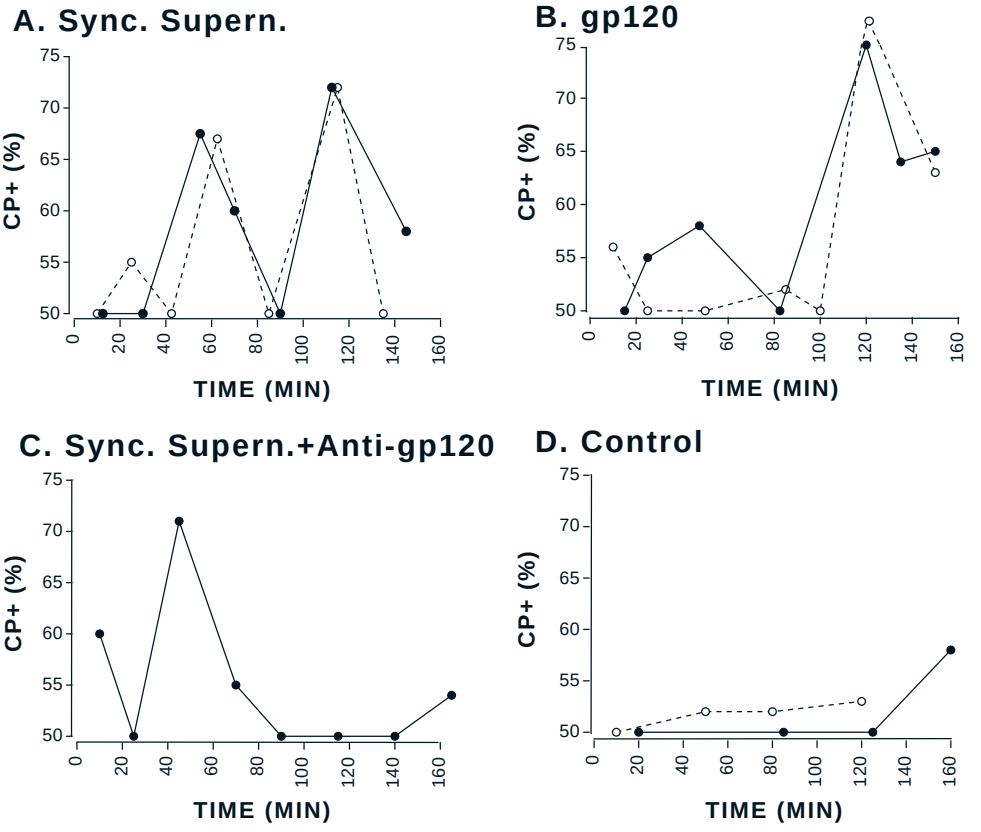

Fig. 3. The chemotactic behavior of SUP-T1 cells and PBTCs over time in molecular gradients generated in the chamber in Fig. 1. The percentage of the cell population exhibiting a net positive chemotactic response is plotted as a function of time, in each case, after the wells of the chamber are filled. (A) uninfected SUP-T1 cell-conditioned medium in the sink well and HIV-induced SUP-T1 syncytium-conditioned medium (Sync. Supern.) in the source well; (B) EBSS alone in the sink well and 1 μg/ml gp120 in EBSS in the source well; (C) uninfected SUP-T1 cell-conditioned medium in the sink well and HIV-induced SUP-T1 syncytium-conditioned medium plus anti-gp120 antibody in the source well; (D) EBSS alone in both sink and source wells (control). Responding SUP-T1 cells, unfilled circles; responding PBTCs, filled circles. All values less than or equal to 50% are plotted as 50%.

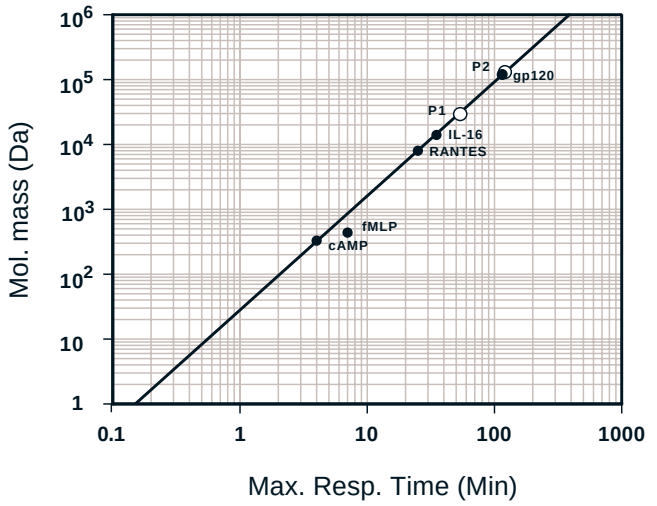

Fig. 4. Estimating the molecular masses of the two chemoattractants in HIV-induced syncytium-conditioned medium. Chemokinetic and chemotactic agents with known molecular masses were individually placed in the source well and buffer was placed in the sink well of the chamber in Fig. 1, and responding cells were placed at the bridge. Different fields of cells were continuously videorecorded for 10 to 30 minutes and this was repeated in sequence through a 150 minute period. Cells were then analyzed by computer-assisted methods for chemotaxis and/or chemokinesis. The maximum response time was the time of maximum chemotactic or chemokinetic response. The maximum response time was plotted as a function of molecular mass. Known molecules (filled circles) and responding cells include the following: cAMP (328 Da), *Dictyostelium discoideum* amoebae; fMLP (438 Da), human polymorphonuclear leukocytes; RANTES (8 kDa), IL-16 (14 kDa), and gp120 (120 kDa), peripheral blood T cells. The maximum response times of the low molecular mass (P1) and high molecular mass (P2) chemoattractants in HIV-induced syncytium conditioned medium (Fig. 3A) were then located on the plot and the molecular masses of each estimated to be ~30 and ~120 kDa, respectively. For the chemokines RANTES and IL-16, maximum response time was taken as the elapsed time after filling of the source and sink wells that the cell population achieves maximum average instantaneous velocity.

Chemoattractant is released only by HIV-induced T cell syncytia

To test whether chemoattractant is released by uninfected SUP-T1 cells, RPMI medium was conditioned for 6 hours with SUP-T1 cells at a cell concentration equivalent in total cell volume to that of the HIV-induced syncytia used to condition medium, and either SUP-T1 cells or PBTCs were used as responding cells. Cell-conditioned medium was then placed in the source well and EBSS in the sink well of a gradient chamber, in the same manner used to test syncytium-conditioned medium. Cell-conditioned medium did not stimulate chemotaxis during a 150 minute period of analysis for either responding SUP-T1 cells or PBTCs (time plots not shown). The average C.I. after 120 minutes was −0.07 and −0.08, and the CP+ was 53% and 43% for SUP-T1 cells and PBTCs, respectively (Table 1).

To test whether the release of chemoattractant is a characteristic of T cell syncytia regardless of the mechanism of syncytium induction, SUP-T1 syncytia were induced in uninfected single cell cultures by polyethylene glycol (Vaughan et al., 1976). The resulting syncytia (PEG-syncytia) were picked and then used to condition RPMI medium for 6 hours at a concentration equal in total volume to that of the HIV-induced syncytia used to condition medium. PEG-syncytium-conditioned medium was then tested with responding SUP-T1 cells in the same manner as HIV-induced syncytium-conditioned medium was tested. PEG-syncytium-conditioned medium did not stimulate chemotaxis during a 150 minute period of analysis (time plot not shown). The average C.I. after 120 minutes was 0.00 and the CP+ 54% (Table 1). These results demonstrate that the release of T cell chemoattractant was specific to HIV-induced syncytia.

The high molecular mass chemoattractant released by HIV-induced T cell syncytia is gp120

The time it took for the two factors in HIV-induced syncytium-conditioned medium to generate gradients which both SUP-T1 cells and PBTCs could assess was consistent with molecular masses of approximately 30 and 120 kDa (Fig. 4). The second of these was similar to the molecular mass of the virally encoded glycoprotein gp120, expressed on the surface of infected cells. Since gp120 is shed by HIV-infected cells in culture (Gelderblom et al., 1985; Schneider et al., 1986), we tested whether soluble gp120 functions as a single cell chemoattractant by adding a solution of 1 μg/ml of gp120 in EBSS to the source well and EBSS alone to the sink well of a gradient chamber, and then monitoring the behavior of individual SUP-T1 cells or PBTCs for 150 minutes. A single CP+ peak was observed at 120 minutes for both responding cell types (Fig. 3B), coincident in time with the second peak of chemotaxis stimulated by HIV-induced syncytium-conditioned medium (Fig. 3A). The time of the response was consistent with the molecular mass of gp120 (Fig. 4). The average C.I. of cells responding to gp120 at 120 minutes was +0.26 for SUP-T1 cells and +0.33 for PBTCs, and the CP+ was 79% for SUP-T1 cells and 81% for PBTCs (Table 1). In Fig. 5A and B, respectively, centroid tracks are presented after 120 minutes of incubation of PBTCs at the bridge of a chamber in which buffer has been placed in both wells and at the bridge of a chamber in which buffer has been placed in the sink and gp120 in the source wells of the chamber. While the tracks of cells in the former case are short and directed in random directions, the tracks of cells in the latter case are longer and, for the most part, directed towards the source well.

To test whether the high molecular mass chemoattractant in syncytia-conditioned supernatant was in fact gp120, polyclonal anti-gp120 antibody was added to syncytium-conditioned supernatant, the mixture incubated for 15 minutes at 37°C, and the mixture placed in the source well of a gradient chamber to assess PBTC chemotaxis. In the presence of anti-gp120 antibody, the first peak of chemotactic responsiveness at 55 minutes still occurred, but the second peak at 120 minutes was abolished (Fig. 3C). The C.I. and CP+ at 120 minutes were depressed to +0.01 and 57%, respectively (Table 1).

The high molecular mass chemoattractant (gp120) functions through interaction with the CD4 receptor

If the high molecular mass chemoattractant in syncytium-conditioned medium is gp120, then the chemotactic receptor on responding T cells should be CD4. To test this possibility, PBTCs were incubated in 4 μg per ml of monoclonal anti-CD4 antibody for 15 minutes at 37°C, and the cells plus antibody

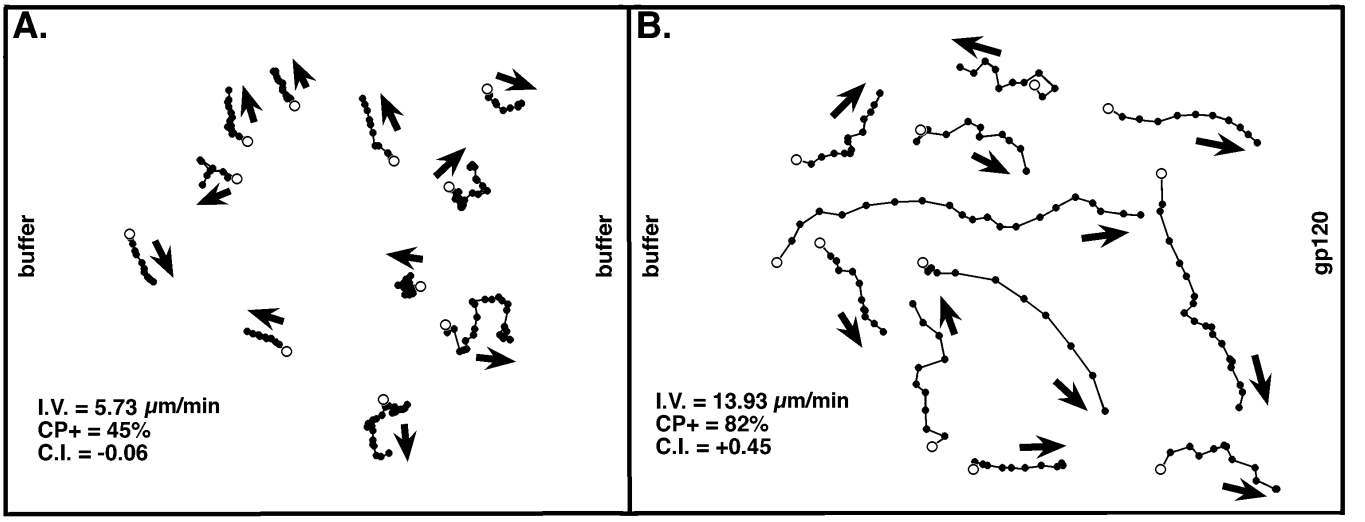

Fig. 5. Examples of the behavior of peripheral blood T cells when both wells of the chamber contain buffer or when the sink well contains buffer and the source well contains 1 μg/ml gp120. Both fields were analyzed at 110-130 minutes after filling the source and sink wells of the chemotaxis chamber. The computed centroid of each cell is plotted at 1 minute intervals over periods of continuous translocation. The centroids are connected to form translocation tracks. The centroid at the beginning of each track is noted by an unfilled circle. The net direction of translocation is noted be an arrow for each track. Over half the cells in a field were motile, and only motile cells are shown. (A) random movement of cells when buffer is placed in both wells. (B) directed movement of cells towards the source of a gradient of gp120. I.V., average instantaneous velocity; CP+, percentage motile cells exhibiting a net positive chemotactic response; C.I., average chemotactic index.

then placed at the bridge of a gradient chamber to assess chemotaxis. Antibody was also mixed with the HIV-induced syncytium-conditioned medium. In the presence of anti-CD4 antibody, the first peak of chemotactic responsiveness at 55 minutes still occurred, but the second peak at 120 minutes was abolished (Table 1). These results not only suggest that the high molecular mass factor (gp120) functions through the CD4 receptor, but also that the low molecular mass factor does not.

gp120 is a chemokinetic as well as chemotactic stimulant

The computer-assisted analysis of single cell behavior in the gradient chamber developed for this study provides not only measures of directed cell movement in the form of the average C.I. and CP+, but also an independent measure of velocity for each individual cell analyzed. In the example of *D. discoideum* amoebae in buffer (Fig. 2A) and in a cAMP gradient (Fig. 2B), the average instantaneous velocity increased from 6.39 μm per minute in the former to 8.54 μm per minute in the latter, and in the example of human PMNs in buffer (Fig. 2C) and in a fMLP gradient (Fig. 2D), the average instantaneous velocity increased from 8.26 μm per minute in the former to 13.88 μm per minute in the latter. These results demonstrate that cAMP and fMLP are chemokinetic as well as chemotactic stimulants. The chemokinetic activity of gp120 as well as that of the low and high molecular mass chemoattractants in HIV-induced syncytium-conditioned medium was, therefore, assessed in the same manner by computing the average instantaneous velocity of cells at peak CP+ in each case, and comparing it to that when EBSS was placed in both wells of the chamber. For SUP-T1 cells, the average instantaneous velocity was 5.2±1.3 μm per minute when analyzed in a chamber in which EBSS was placed in both wells (Table 2). These values remained relatively constant through 120 minutes of analysis in the chamber (time

Table 2. Chemokinetic response of T cells to HIV-induced syncytium-conditioned medium and gp120*

Cell type	No. cells analyzed	Chamber*		Period of analysis (min)	Instantaneous velocity (μm/min)†
		Sink	Source		
SUP-T1	38	EBSS	EBSS	100-130	5.2±1.3
	58	EBSS	gp120	110-130	6.9±2.1
	18	Cond.med.	H-Sync.supern.‡	50-70	5.7±2.0
	30	Cond.med.	H-Sync.supern.§	110-130	7.8±3.5
(CD4+) PBTC	31	EBSS	EBSS	100-130	6.8±3.6
	21	EBSS	gp120	115-135	15.0±5.0
	19	Cond.med.	H-Sync.supern.‡	45-65	9.6±3.5
	20	Cond.med.	H-Sync.supern.§	110-130	12.2±4.8

*Explanation of abbreviations can be found in Table 1, footnote *.
†Mean ± standard deviation.
‡Early peak of chemotaxis (resolved from time plots like the ones in Fig. 3).
§Later peak of chemotaxis (resolved from time plots like the ones in Fig. 3).

plot not shown). In a gradient of gp120, the instantaneous velocity of SUP-T1 cells was 6.9±2.1 μm per minute (Table 2). The behavioral difference between buffer and a gradient of gp120 was statistically significant ($P<0.001$). The instantaneous velocity of SUP-T1 cells responding to gradients of the low or the high molecular mass chemotactic factor in HIV-induced syncytium-conditioned medium was 5.7±2.0 and 7.8±3.5 μm per minute, respectively (Table 2). The difference between the velocity in buffer and that in the gradient of the high molecular mass attractant was significant ($P<0.001$), suggesting that at least the high molecular mass attractant is a chemokinetic as well as a chemotactic stimulant.

More pronounced results were obtained with responding PBTCs (Table 2). While the average instantaneous velocity of these cells in EBSS was 6.8±3.6 μm per minute, the average instantaneous velocity in a gradient of gp120 was 15.0±5.0 μm per minute (Table 2). The difference between the former and latter was again significant ($P<0.001$). The average instantaneous velocity in gradients of the low and high molecular mass chemotactic factors in HIV-induced syncytium-conditioned medium was 9.6±3.5 and 12.2±4.8 μm per minute, respectively (Table 2). Again, the difference in the velocity in buffer and in a gradient of the high molecular mass chemoattractant was statistically significant ($P<0.001$), suggesting that the high molecular mass chemoattractant was strongly chemokinetic. In Fig. 5A and B, examples are presented of fields of PBTCS in buffer alone or in a gradient of gp120, respectively. It is evident that the distances between the centroids of cells in the cell tracks are shorter on average in buffer, and subsequently, the tracks are more truncated. These results demonstrate that gp120 is both a chemotactic and chemokinetic stimulant.

DISCUSSION

Syncytia induced by HIV in cultures of both SUP-T1 cells and peripheral blood T cells are neither disorganized nor functionless, but, rather, are motile, and actively mimic the subcellular organization of single cells (Sylwester et al., 1993, 1995; Shutt et al., 1995; Soll and Kennedy, 1994). HIV-induced T cell syncytia formed in culture crawl through extension of a single giant pseudopod with the same velocity, directionality, and behavior cycle as single cells. Syncytia of the SUP-T1 cell line have been demonstrated to phagocytose as well as fuse with T cells, again through extension of a giant pseudopod (Murphy et al., 1995). Their capacity to extend a giant pseudopod and to crawl also make them both destructive and invasive. When placed on hydrated collagen, they disorganize the gel through the extension and retraction of giant pseudopods, and when placed on bovine aortic endothelium, they generate large holes, primarily at the site of giant pseudopod extensions (Sylwester et al., 1998). Finally, motile syncytia have been demonstrated in the lymph nodes of HIV-infected individuals (D. C. Shutt, J. T. Stapleton, J. A. Goeken, A. W. Sylwester, G. P. Kealey, P. Cederna, R. C. Kennedy and D. R. Soll, unpublished).

In our original studies of syncytium behavior (Sylwester et al., 1993, 1995, 1997; Shutt et al., 1995a; Soll, 1997), we performed long term video analyses of fields of cells containing syncytia, and noticed that in some monitored fields, single cells and small syncytia moved in a directed fashion towards large syncytia, across distances too great to be accounted for by pseudopodial or filopodial interactions. We, therefore, sought a method for testing whether HIV-induced syncytia released a chemoattractant. In previous analyses of the chemotactic activity of a number of cytokines (Schall et al., 1990; Schall, 1991; Center and Cruikshank, 1982; Cruikshank and Center, 1982; Larsen et al., 1989) as well as gp120 (Kornfeld et al., 1988), micropore filter assays, first introduced by Boyden (1962), were used to test for chemotactic activity. In these studies, the majority of tested chemokines were judged to be chemoattractants. In the micropore filter assay, cell migration is assessed across a porous membrane separating two reservoirs, one containing the supporting medium lacking attractant and the other containing medium with attractant (Wilkinson, 1988). The cells in a test chamber are initially placed on the side of the membrane in buffer or medium lacking test substance. At a set time, well after the presumed development of the gradient, the accumulation of cells on the opposite side of the membrane is measured. Alternatively, the number of cells that have migrated beyond a certain depth in the filter at a fixed time is determined in a filter treated with cell fixative. These methods do not allow for the direct observation of single cell behavior during migration. Therefore, the behavior of individual cells in a population is unknown, and at the end of the assay, the accumulation of a small fraction of the population is assessed on or toward the side of the membrane bordering the well containing attractant. Although in many studies employing the micropore filter assay, a checkerboard analysis is applied (Zigmond and Hirsch, 1973) in which cell migration is assessed under conditions in which attractant is placed in both chambers, in neither chamber, only in the upper chamber, or only in the lower chamber, the discrimination between chemotactic versus chemokinetic stimulation is, as Wilkinson (1988) has noted, ‘at best, inferential’. Zigmond (1978) has pointed out that the mechanism of an apparent chemotactic response cannot really be determined in a micropore filter assay, since the results cannot discriminate between an increase in the proportion of responsive cells, a change in the accuracy of orientation, a change in the frequency and/or magnitude of turning, or a change in the rate of translocation in a particular direction. As suggested by Zigmond and Hirsch (1973), the checkerboard correction should be calculated by first determining the velocity of the cell populations in each concentration of attractant used in the micropore filter assay. Unfortunately, this mathematical correction factor is under-utilized (Wilkinson, 1988). Rhodes (1982) has argued further that the checkerboard correction falsely assumes that the behavior of cells crawling through a gradient is the same as the average behavior of cells crawling in static concentrations of chemoattractant that are equivalent to the beginning and ending concentrations in a gradient.

We, therefore, decided to test for a chemoattractant in HIV-induced syncytium conditioned medium by using a chamber in which all cells in a population can be individually analyzed. Such chambers (Zigmond, 1977, 1978) provide measurements of single cell velocity and direction, providing unequivical discrimination between chemotaxis and chemokinesis, and information on the proportion of cells in the population undergoing positive chemotaxis. To accomplish this, a chamber was developed which allowed conditioning of the migration surface and which was encapsulated so that there was no

evaporation from the chamber during long periods of analysis, which proved necessary for the long time periods it took to establish gradients of high molecular mass molecules (Lauffenberger et al., 1988). The efficacy of this chamber was demonstrated using two cell types previously demonstrated to respond in a true chemotactic fashion to chemoattractants of known molecular masses. Using this chamber, we have demonstrated that HIV-induced syncytia release factors which function as bona fide CD4+ T cell chemoattractants. Since neither uninfected SUP-T1 cells nor SUP-T1 syncytia induced by polyethylene glycol release detectable chemoattractant, the release of chemoattractants appears to be a specific characteristic of HIV-induced T cell syncytia. Two chemotactic factors were demonstrated in HIV-induced syncytium-conditioned medium, one with an estimated molecular mass of approximately 30 kDa and one with an estimated molecular mass of approximately 120 kDa. The latter one has approximately the same molecular mass as the HIV-1 *env* gene product gp120, which has been demonstrated to leak from virus and infected cells in culture (Gelderblom et al., 1985; Schneider et al., 1986). We have demonstrated that soluble gp120 functions as a chemoattractant for both SUP-T1 cells and PBTCs verifying prior results based on the micropore filter assay (Kornfeld et al., 1988), and that the addition of anti-gp120 antibody selectively blocks the activity of the high molecular mass component of synctium-conditioned medium, suggesting that the latter component is gp120. The addition of anti-CD4 antibody also selectively blocks the activity of the high molecular mass component of syncytium-conditioned medium, suggesting that the high, but not the low, molecular mass component requires the CD4 receptor. These latter results suggest that the CD4 receptor functions as a chemotactic receptor and that the low molecular mass attractant is not a cleavage product of gp120, since it does not require the CD4 receptor. Although we have little information on the identity of the low molecular mass attractant released by HIV-induced syncytia, the fact that anti-gp120 antibody does not block its activity also suggests it is not a cleavage product of gp120. In addition, the speed at which it established a gradient, which was consistent with a molecular mass of approximately 30 kDa, suggests it is not the virally encoded protein gp41. The virally encoded Tat protein has been reported to have chemoattractant activity using a micropore filter method (Mitola et al., 1997), but at 19 kDa it is too small to account for the low molecular mass peak we observe.

The release of two chemoattractants by HIV-induced syncytia explains our sporadic observation that single cells sometimes move in a directed fashion towards large syncytia in HIV-infected SUP-T1 cell cultures. The release of chemoattractants by syncytia could potentially play a pathogenic role in vivo. If fusion proves to be a significant avenue of T cell death in HIV-infected patients, the release of chemoattractants by HIV-infected cells and syncytia would facilitate fusion and, therefore, T cell death. Recently, it was demonstrated that initial decreases in peripheral blood CD4+T cell populations are not necessarily paralleled by concomitant decreases in the population of CD4+ T cells in lymphoid tissue (Røsok et al., 1996). Since lymph nodes are the major repositories of virus and infected cells, the lymph node very likely contains the highest concentration of soluble gp120. The genesis of gradients of gp120 and the lower molecular mass chemoattractant could, potentially, play a role in attracting circulating T cells to lymphoid tissue and retaining them there for their ultimate infection and demise in the progression of this disease.

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