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Human Immunodeficiency Virus Type 1 Strains of Subtypes B and E Replicate in Cutaneous Dendritic Cell–T-Cell Mixtures without Displaying Subtype-Specific Tropism

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A report that genetic subtype E human immunodeficiency virus type 1 (HIV-1) strains display a preferential tropism for Langerhans cells (epidermal dendritic cells [DCs]) compared to genetic subtype B strains suggested a possible explanation for the rapid heterosexual spread of subtype E strains in Thailand (L. E. Soto-Ramirez et al., Science 271:1291–1293, 1996). In an independent system, we applied subtype E and B isolates to skin leukocytes, since skin is a relevant model for the histologically comparable surfaces of the vagina and ectocervix. Isolates of both HIV-1 subtypes infected DC–T-cell mixtures, and no subtype-specific pattern of infection was observed. Purified DCs did not support the replication of strains of either subtype B or E. Our findings do not support the conclusion that subtype E strains have a preferential tropism for DCs, suggesting that other explanations for the rapid heterosexual spread of subtype E strains in Asia should be considered.

Heterosexual transmission now accounts for most cases of human immunodeficiency virus type 1 (HIV-1) infection worldwide (6, 38). During transmission, HIV-1 virions or infected cells are brought into contact with the genital mucosa or with rectal or oral tissues. It is important to understand how HIV-1 interacts with these tissues, since an effective vaccine may need to prevent the very earliest stages of the infection process. In particular, dendritic cells (DCs) of the vaginal epithelium have been implicated as a possible initial site of infection with HIV-1 and simian immunodeficiency virus (41, 42, 48, 53, 63).

DCs are found throughout the body, in the epithelia, blood, lymph, and lymphoid and nonlymphoid parenchymal tissues (64). Their major function is to recognize foreign antigens and transport them via the afferent lymphatic system to the lymphoid tissues (2, 64), where they initiate T-cell responses (5, 26, 34, 61). The exposed surfaces of the pharynx, vagina, cervix, and anus are similar to the skin in both histology and DC content (13, 43, 44). DCs have also been identified within the rectal or colonic mucosa, particularly within lymphoid follicles located in the submucosal regions (20, 23, 51). Because skin is readily available, the biological features of DCs have been studied extensively in this tissue, which has also been used as a model for the body surfaces involved in HIV-1 transmission because of the histopathological similarities among these tissues (55, 56, 62). DCs are likely to be among the first cells infected by HIV-1 following exposure of genital epithelial surfaces to the virus during sexual contact with an infected individual (63). HIV-1-carrying DCs could then migrate to the lymphoid tissues, interact with T cells, establish a productive infection, and spread HIV-1 systemically. Although this sequence of events is difficult to demonstrate in vivo, studies using skin DCs as an in vitro model system suggested that these cells could remain infectious for up to 1.5 days after encountering HIV-1 (56). The HIV-1-pulsed DCs then initiated a robust infection upon interacting with $CD4^+$ T cells, indicating that very small numbers of infected DCs are sufficient to propagate large amounts of new virus in the appropriate cellular environment (56). This is consistent with the above-outlined hypothesis for what happens during sexual transmission of HIV-1 in vivo but clearly does not prove it.

To date, 10 HIV-1 genetic subtypes (A through I and group O) have been designated on the basis of *env* or *gag* sequences (30, 35, 37, 40, 46, 65, 70). HIV-1 strains of subtypes B and E were independently introduced into Thailand in the late 1980s (28, 40, 49, 69). The B subtype is presently found predominantly, but not exclusively, in intravenous drug user (IVDU) populations, while subtype E strains have spread rapidly in the general heterosexual population, including IVDU cohorts. In recent years, the proportion of new subtype E HIV-1 infections in Thailand has increased inexorably (27, 28). This has been taken to indicate that the subtype E strains are inherently more transmissable, specifically by heterosexual contact (31). However, epidemiological factors, such as founder effects and the relatively closed nature of subtype B-infected IVDU communities, may also contribute to the apparent compartmentalization and the relatively restricted spread of subtype B strains in Thailand (39). The hypothesis that subtype B strains are difficult to transmit heterosexually is also not supported by the burgeoning subtype B epidemics in heterosexual populations of the Caribbean and South America (6, 19, 33, 38, 39).

Soto-Ramirez et al. (62) have reported that highly enriched Langerhans cells (LCs; epidermal DCs) preferentially support the replication of subtype E strains compared to strains of

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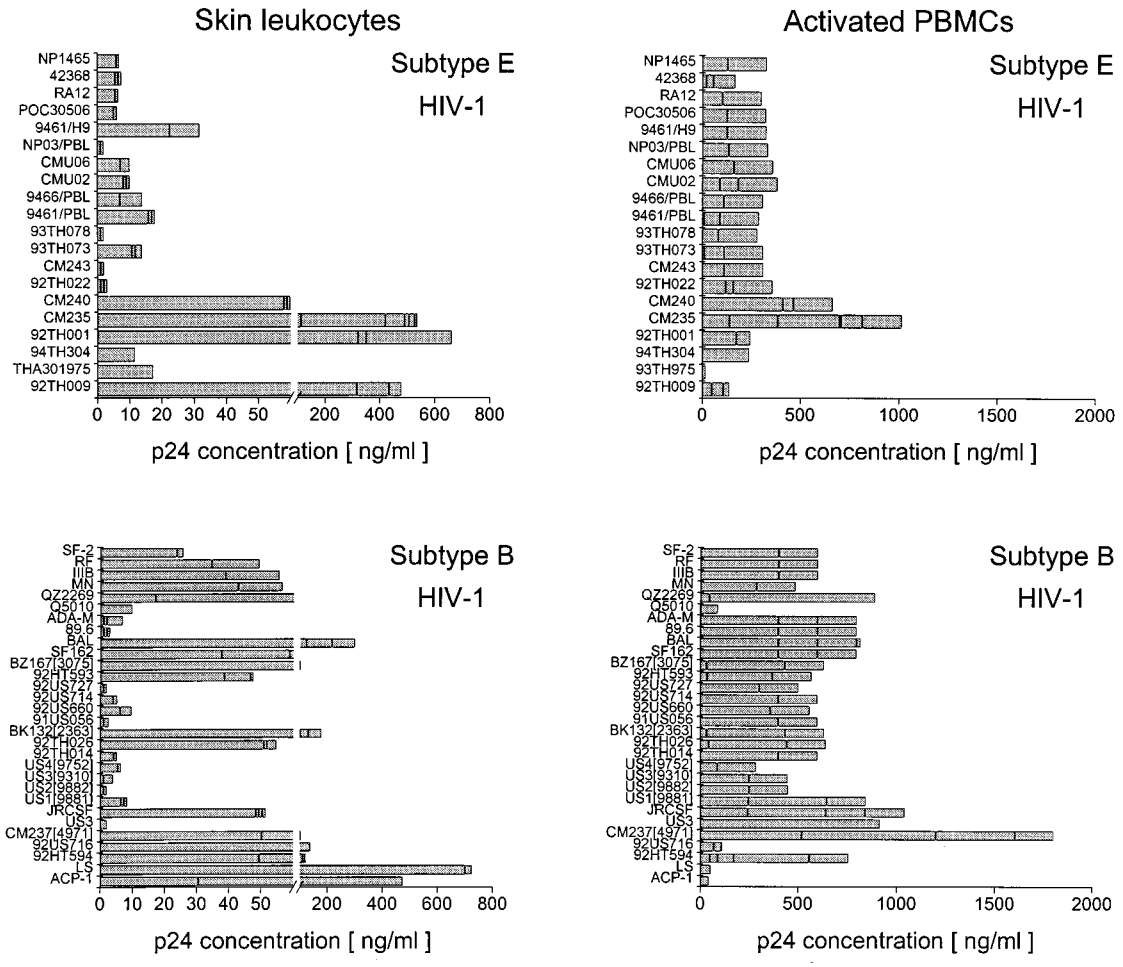

FIG. 1. Replication of subtype B and E HIV-1 isolates in skin DC–T-cell mixtures and PBMCs. Activated PBMCs and skin-derived DC–T-cell mixtures were exposed to an extensive panel of subtype E and B HIV-1 isolates and cultured for up to 3 weeks. Infections were monitored by an enzyme-linked immunosorbent assay measuring the release of p24 antigen into the culture supernatants. The peak p24 levels (in nanograms per milliliter) obtained from all experiments are presented, with the black vertical markings within each bar denoting individual p24 values obtained for that particular isolate in the multiple experiments.

subtype B. This selective tropism of subtype E viruses for DCs of the genital mucosa was suggested to provide a biological explanation for the apparently increased efficiency of heterosexual transmission of subtype E strains (62). However, our experience is that purified populations of DCs are not readily infectable with HIV-1 (subtype B) and that productive infection also requires the presence of $CD4^+$ T cells (55, 56). To see if we would observe different results with subtype E strains, we exposed highly purified skin DCs or DC–T-cell mixtures to a selection of HIV-1 strains of both subtypes B and E.

Replication of HIV-1 subtypes B and E in skin DC–T-cell mixtures. We initially examined the replication of a large panel of HIV-1 isolates of subtypes B and E in the permissive DC–T-cell milieu that emigrates from normal human skin in organ culture (54, 56, 59, 60). The isolates tested and their ability to replicate in this system are summarized in Fig. 1. Virus isolates were obtained from the U.S. Military HIV Research Program (70) and the National Institutes of Health AIDS Research and Reference Reagent Program (45). The subtypes of isolates that were examined at the Walter Reed Army Institute of Research were determined by DNA sequencing of the *env* gene or by heteroduplex mobility assay (1, 40). Note that most isolates were macrophage-tropic (M-tropic) primary isolates, but a few T-cell-line-adapted (TCLA) viruses (IIIB, RF, MN, and SF-2), as well as one dual-tropic virus (89.6), were also used for comparison. The TCLA and dual-tropic viruses were all of subtype B.

Cutaneous DC–T-cell mixtures, containing DCs, T cells, and DC–T-cell conjugates, were obtained from normal split-thickness skin obtained from cadaver donors as previously described (54, 56). In brief, skin was placed into organ culture, and after 3 to 4 days the migrated cells were treated with collagenase D and collected. Cadaver skin specimens authorized for research were provided by the New York Firefighters' Skin Bank, New York Hospital, New York, N.Y., and Lifenet, Virginia Beach, Va. Peripheral blood mononuclear cells (PBMCs) were prepared from normal buffy coats obtained from the New York Blood Bank. Activated T blasts were generated by culturing the PBMCs with 5 ng of staphylococcal enterotoxin E superantigen (Toxin Technology) per ml for 2 to 3 days or by stimulating them with 1 μg of phytohemagglutinin per ml overnight.

Skin cell emigrants and activated PBMCs were exposed to HIV-1 isolates of subtypes B and E (30 to 300 50% tissue culture infectious doses [$TCID_{50}$] or 4 to 20 ng of p24 per ml per 10^5 cells in round- or flat-bottomed 96-well trays [Flow/

TABLE 1. Infection of skin leukocytes and activated PBMCs with E and B subtype isolates[a]

Virus	Subtype	Cell type[b]	HIV-1 replication in cell cultures on day[c]:				
			3	5	10	13	17
CM235	E	Skin	<0.01	7.6	20.6	15.0	11.5
		Blood	2.0	6.7	8.1	7.8	4.8
93TH073	E	Skin	<0.01	1.8	10.6	10.7	8.0
		Blood	0.1	1.9	3.9	10.7	7.8
9461	E	Skin	<0.01	1.6	10.5	15.4	15.8
		Blood	<0.08	1.9	1.9	8.2	5.9
CMUO2	E	Skin	<0.01	3.4	8.0	5.8	2.8
		Blood	0.6	88.4	2.6	6.3	4.1
42368	E	Skin	<0.01	<0.5	3.7	5.5	5.1
		Blood	<0.1	9.1	1.9	2.3	18.5
BK132	B	Skin	<0.01	11.2	50.6	>100	>100
		Blood	0.6	5.6	25.3	34.7	22.9
US1	B	Skin	<0.01	0.7	5.9	6.4	5.6
		Blood	<0.01	1.9	12.1	53.7	247.4
92TH026	B	Skin	<0.01	1.8	20.8	42.4	50.9
		Blood	0.3	1.9	14.9	32.9	43.2
92HT593	B	Skin	<0.01	2.1	20.4	37.8	38.6
		Blood	<0.01	1.9	1.9	16.5	36.2
BZ167	B	Skin	<0.01	49.0	223.3	>100	>100
		Blood	1.8	3.5	17.2	33.6	30.5

[a] Skin leukocytes and activated PBMCs were exposed to various isolates of HIV-1 (20 ng of p24 equivalents added per ml per 10^5 cells), and after overnight culture the cells were washed and recultured for another 17 days. Culture supernatants were harvested at the indicated times.
[b] Skin, skin leukocytes; blood, PBMCs.
[c] HIV-1 replication was assessed by p24 antigen determination (nanograms per milliliter).

ICN]). After incubation for 1.5 h or overnight at 37°C, the cells were washed three times (56) and cultured at 37°C. Culture supernatants were collected over the next 2 to 3 weeks and monitored for p24 antigen production by an in-house enzyme-linked immunosorbent assay (47) or with the Coulter (Hialeah, Fla.) p24 antigen kit.

The replication (as measured by p24 antigen production) of five subtype E and five subtype B primary isolates in a single preparation of activated PBMCs and of a skin DC–T-cell mixture is recorded in Table 1, and the peak p24 levels measured in 15 similar experiments are summarized in Fig. 1. In each of these experiments at least 4 isolates (2 E subtype and 2 B) were compared in both skin and PBMC cultures, and in some experiments over 40 isolates were compared (when cell numbers permitted this). Most isolates were tested at least twice. Some isolates (of both subtypes B and E) did not replicate well in the skin leukocytes but were still able to infect activated PBMCs, which generally produced more p24 antigen (Fig. 1). In some experiments, HIV-1 strains of both subtypes were unable to infect the skin DC–T-cell mixtures when the inoculum was small (30 $TCID_{50}$ or 4 ng of p24 per ml per 10^5 cells). However, increasing the inoculum by three- to fivefold usually resulted in at least a low level of infection by all the strains tested.

It is clear that there was strain-specific replication in the cutaneous DC–T-cell milieu, but this was independent of the genetic subtype of the test strains. Some subtype E strains (e.g., CM240, CM235, and 92TH001) replicated well in the DC–T-cell mixtures and some (e.g., 93TH078, CM243, and 92TH022) replicated poorly, but no pattern was discernible that would be consistent with a preferential tropism of subtype E strains for LCs. On average, the primary isolates of subtype B replicated to higher titers than the subtype E strains, but this was true in both PBMCs and the skin DC–T-cell mixtures (Fig. 1). Some HIV-1 strains did replicate better in skin DC–T-cell mixtures than in PBMCs, but again this was true of both B (e.g., LS and ACP-1) and E (e.g., 92TH009 and 92TH001) subtype viruses. The replication of the TCLA or dual-tropic subtype B strains was not notably different from that of the primary viruses in either cell system (Fig. 1).

HIV-1 strains of subtypes B and E require DCs and T cells to replicate. Initial studies demonstrated that many different HIV-1 isolates could replicate in the permissive DC–T-cell environment in a subtype-independent manner. However, it seemed possible that subtype-specific differences in infectability would become apparent when purified T cells and DCs were examined separately.

DCs are known to promote vigorous replication of HIV-1 (7). In vivo, heavily infected syncytia derived from DCs were identified at the mucosal surface of the nasopharyngeal lymphoid organs of HIV-1-infected individuals (21, 22). However, in vitro studies are contradictory with respect to the ability of populations of both skin-derived (4, 10, 36, 55–58, 62) and blood-derived (7–9, 29, 32, 50, 52, 68) DCs to support virus replication. These discrepancies may be attributable at least in part to the different purification methods employed and the subsequent purity of the DCs (7). There is no question, however, that the DC–T-cell environment is extremely permissive for virus replication (3, 8, 9, 55–57).

Skin-derived DCs and T cells were separated by cell sorting techniques (54–56). Purified fractions were usually >98% pure as determined by fluorescence-activated cell sorter analysis and immunocytochemistry (54, 55). The emigrated DC–T-cell mixture, purified DC and T-cell fractions, and mixtures in which the sorted DCs and T cells were recombined (6×10^4 T cells per 3×10^4 DC) were each separately exposed to HIV-1 (100 $TCID_{50}$ per 10^5 cells) and cultured for up to 2 weeks. Reverse transcriptase activity (55) and p24 levels (as described above) were measured in the culture supernatants. The anti-HIV-1 p24 monoclonal antibody (hybridoma 183, clone H12-5C (11); The AIDS Research and Reagent Program) was used to detect p24 antigen at the single-cell level by immunoperoxidase staining cytospin preparations for p24 (54, 56). Due

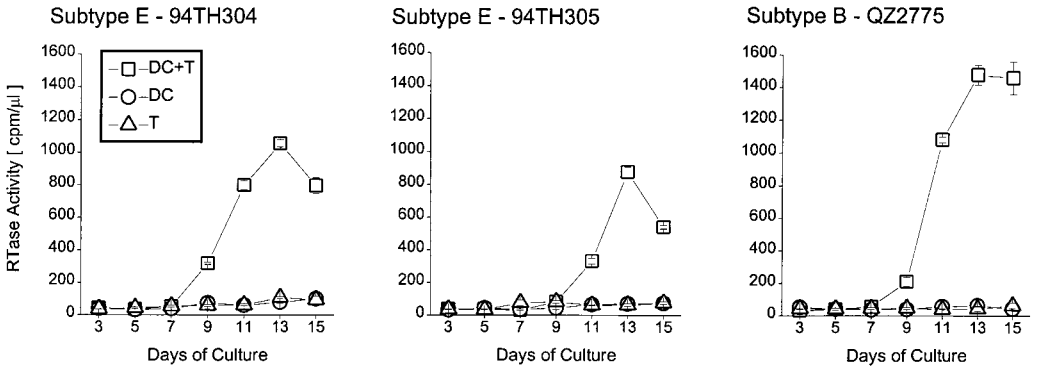

FIG. 2. Purified cutaneous DCs do not support the growth of HIV-1 of subtype B or E. Aliquots of 10^5 sorted DCs (DC), 10^5 sorted T cells (T), and mixtures of the two (DC+T; 1:2) were exposed to 100 $TCID_{50}$ of the indicated HIV-1 isolate and cultured for 15 days. Productive infection was assessed by monitoring the culture supernatants for the presence of reverse transcriptase (RTase) activity (mean counts per minute ± standard errors of the means of triplicate samples are shown). The results from one of four similar experiments are depicted.

to cell number restraints, only some of the isolates listed in Fig. 1 could be examined in these cultures (six B and six E subtype strains in total). As had been found previously (55, 56), neither DCs nor T cells alone could support HIV-1 replication when highly purified, and this was true for both B and E subtype strains (Fig. 2; Table 2). However, the reconstituted mixtures of DCs and T cells could be efficiently infected with all the B and E subtype viruses tested, leading to both single cells and multinucleated syncytia that stained positively for p24 antigen (Fig. 3).

Contamination of enriched DC populations with a small proportion of T cells permits active HIV-1 replication. In three separate experiments in which seven different isolates (three E and four B subtype strains) were tested, we noted that populations of highly enriched DCs could be productively infected by both subtype B and E strains (Fig. 3; Table 3). The cultures appeared to contain >98% DCs at the time of HIV-1 addition (as determined by fluorescence-activated cell sorter analysis and/or immunohistochemical analysis) (data not shown), but a robust HIV-1 infection was observed within a few days (Fig. 3 shows an example of infection with a subtype E strain; infections with subtype B strains were qualitatively similar [data are not shown]). However, when the infected putative DC cultures were stained for the CD3 T-cell marker, $CD3^+$ single cells and syncytia were evident (Fig. 3). Clearly, the degree of purity was less than had been initially assessed. Less frequently, we observed that a small number of DCs contaminating the T-cell preparations was sufficient to support a robust replication of HIV-1 (Tables 2 and 3). Together, these results confirm our previous findings that T cells collaborate with DCs to promote HIV-1 replication (55, 56), and they indicate that even when very few T cells contaminate the highly enriched DC environment (or vice versa), this can be sufficient to enable vigorous virus replication to occur. However, once again, we could detect no subtype-specific pattern of HIV-1 replication in DCs or DC–T-cell mixtures.

As indicated above, there are discrepancies in the literature regarding the infectability of cutaneous DCs (4, 10, 36, 55–58, 62) which may be attributable to the final purity of the DC population (Fig. 3 and Table 3), as well as to differences in the culture conditions and methods of isolation (7). We note that the method that we used to prepare DCs differs from that employed by Soto-Ramirez et al. (62). However, it is unlikely that these methodology differences are sufficient to account for the discrepancies between the two sets of observations. Had LCs possessed an intrinsically greater potential to replicate E subtype strains, we would have expected our experiments to detect this subtype-specific tropism. Furthermore, using a slightly different system again, but one similar to that described by Soto-Ramirez et al. (62), Dittmar et al. also report no evidence of subtype-specific replication of HIV-1 in LCs (16).

A second difference between our results and those of Soto-Ramirez et al. is that the latter observed efficient HIV-1 replication in apparently pure LC populations (62) whereas we did

TABLE 2. Peak HIV-1 p24 production in cutaneous-cell subsets

Expt	Virus	Subtype	Concn of p24 produced (ng/ml) in:		
			DCs	T cells	DCs + T cells
1	92TH009	E	3.5	<1	45.2
	92HT594	B	2.0	4.7	40.6
	92US716	B	3.0	3.1	42.3
2	92TH001	E	<1	7.8	321.3
	CM235	E	<1	25.1[a]	113.1
	LS	B	<1	4.3	700.7
3	92TH001	E	<1	1.2	28.8
	ACP-1	B	<1	<1	30.5
	Q5010	B	<1	<1	9.9
	QZ2269	B	<1	<1	14.9

[a] Large $HLA\text{-}DR^+$ contaminating DCs were detected in this infected culture.

TABLE 3. Peak p24 production by less pure preparations of skin DCs and T cells

Expt	Virus	Subtype	Concn of p24 (ng/ml) produced by:		
			DCs[a]	T cells[a]	DCs + T cells
5	92TH009	E	50.0	20.0	60.5
	92HT594	B	7.5	<1	12.5
6	92TH009	E	79.9	51.8	317.3
	92TH304	E	<1	<1	11.7
	92US716	B	19.6	1.0	75.1
7	ACP-1	B	92.5	<1	54.8
	QZ2269	B	163.3	<1	45.4
	BAL	B	125.5	<1	82.7

[a] Large $HLA\text{-}DR^+$ and $CD3^+$ cells plus syncytia were evident in the T-cell and DC cultures, respectively, when significant p24 levels were detected.

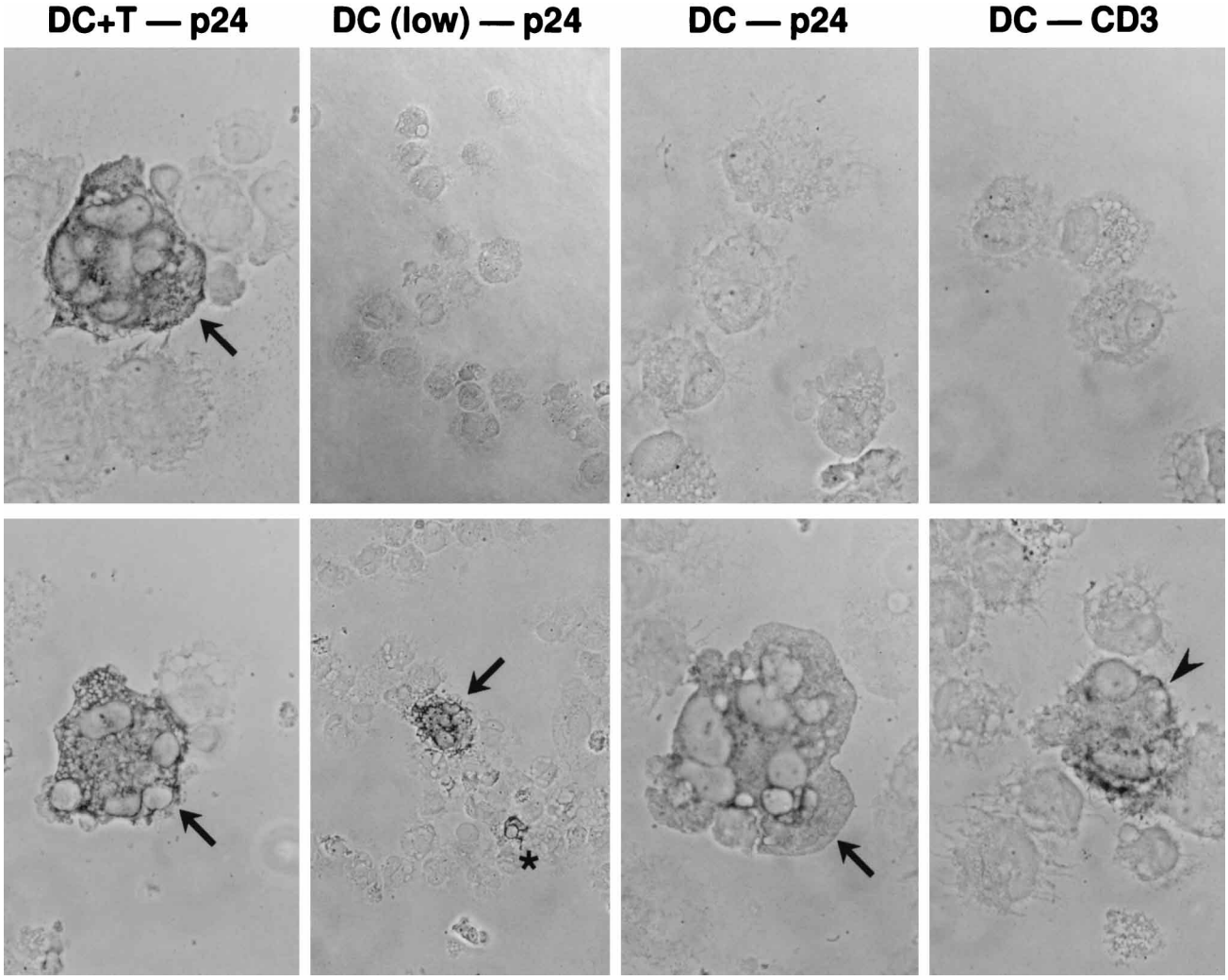

FIG. 3. Very few T cells are sufficient to promote HIV-1 replication in a highly enriched DC environment. Sorted DCs (DC) and the original mixture of DCs and T cells (DC+T) were exposed to the subtype E isolate 92TH009, as described in the legend to Fig. 2, and cytospin preparations were made 4 to 6 days after exposure. The cells were immunoperoxidase stained (dark reaction product) for the HIV p24 protein (p24) and the T-cell marker CD3 (CD3). Data from two separate experiments are shown, and experiment with a highly purified DC preparation (top row) and one in which the DCs were contaminated with $CD3^+$ T cells (bottom row). Syncytia positive for HIV-1 p24 antigen are highlighted with arrows, a $p24^+$ lymphocyte is indicated with an asterisk, and a $CD3^+$ syncytium is shown with an arrowhead. Magnifications: DC(low)—p24, ×200; others, ×500.

not. Although T cells are rare in the epidermis, we have found that a few contaminating T cells (present as single cells or, more importantly, bound to the DCs [54–56]) can be sufficient to permit vigorous HIV-1 replication in DC cultures that appear to be homogeneous when infection is initiated (Fig. 3; Table 3). As we stated above (Fig. 3; Table 3), however, the presence of these T-cell contaminants can often be detected only when the cultures are carefully examined retrospectively, once a robust HIV-1 infection is confirmed. However, again we did not observe any HIV-1 replication patterns that were subtype dependent, even when the issue of T-cell contamination was taken into account. Even though the LC-enriched suspensions studied by Soto-Ramirez et al. (62) were reported to be free of T cells, examination of the infected cultures would be critical for verification, since contamination by T cells is not always immediately apparent (Fig. 3). Such an examination was not reported (62). Dittmar et al. did report that there was infection of LC preparations by different HIV-1 isolates, albeit to a much lesser extent than in activated PBMCs and without any indication of subtype-specific tropism (16). However, this could again reflect a minimal level of T-cell contamination (although the LCs were reported to contain <2% T cells), or it could be a reflection of the cytokine conditions under which the LCs were cultured (16).

It is now clear that HIV-1 requires coreceptors to enter $CD4^+$ T cells (12, 14, 15, 17, 18, 25, 66, 71) and DCs (24). The most important of these for the primary, M-tropic strains of the non-syncytium-inducing phenotype that are most frequently transmitted sexually is CCR5 (25). Blood- and skin-derived DCs both contain mRNA for both CXCR4 and CCR5 (16), and the ligands for these receptors can block TCLA and M-tropic viral entry, respectively, into these DCs (24). Of note in the context of this study is that M-tropic primary strains of subtypes B and E (and also other subtypes) can use CCR5 for entry (12, 71) and that gp120 molecules from both subtypes B and E interact with CCR5 (66). Some strains of both subtypes

can also use CCR3 in transfected cells (12). At least in broad outline, the mechanism of HIV-1 entry into its target cells is probably independent of genetic subtype.

In conclusion, our findings do not support the idea that HIV-1 strains of subtype E are more transmissible between heterosexuals because they have an unusual capacity to replicate in LCs. There can be considerable variation in the ability of individual HIV-1 strains to replicate in DC–T-cell mixtures, suggesting that some strains might be more transmissable than others (just as there is variation in virulence among strains), but we can find no evidence that this variation is subtype dependent (at least for strains of subtypes B and E). A similar conclusion was reached in an independent study using isolates of subtypes A, B, D, E, and F (16). Clearly, all ex vivo and in vitro systems have inevitable technical limitations, and great care should be taken when making claims of transmission probabilities in vivo solely on the basis of in vitro data. It is possible that the spread of subtype E strains in Thailand is due to other, as yet undefined biological factors not examined in this study. Alternatively, the observed patterns of spread of subtype E may be due solely to social and epidemiological factors (19, 38, 39, 67).

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