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CD4 molecules are restricted to the basolateral membrane domain of in vitro differentiated human colon cancer cells (HT29-D4)

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The CD4 glycoprotein serves as a receptor for the human immunodeficiency virus HIV, the etiologic agent of acquired immunodeficiency syndrome (AIDS). We have examined the expression of CD4 molecules in a clone (HT29-D4) derived from a human colon adenocarcinoma cell line. HT29-D4 cells synthesized a 60 kDa polypeptide immunoprecipitated with two anti-CD4 monoclonal antibodies after metabolic or cell surface labeling. This 60 kDa polypeptide was also immunodetected using the same antibodies in human acute lymphoblastic leukemia cells CEM which are known to express CD4. HT29-D4 cells can be induced to differentiate into enterocyte-like cells by removing glucose from the culture medium. Under these conditions, HT29-D4 cells form a polarized epithelial monolayer in which tight junctions separate the plasma membrane in an apical and a basolateral domain. The localization of CD4 molecules in differentiated HT29-D4 cells was exclusively restricted to the basolateral membrane domain as demonstrated by radioimmunoassay and indirect immunofluorescence studies. Therefore the HT29-D4 clonal cell line represents a unique model for polarized HIV infection of colonic epithelial cells and may be useful to understand some of the gastrointestinal disorders occurring in AIDS patients.

1. INTRODUCTION

CD4 is a glycoprotein of approximately 60 kDa expressed on the cell membrane of a helper/inducer subset of human T lymphocytes [1] and, to a lesser extent, on cells of the monocyte/macrophage lineage [2] and on eosinophils [3]. Despite its demonstrated role in T-cell-activation, little is known about the biology of CD4. Several lines of evidence suggest that CD4 binds to the class II MHC antigen HLA-DR thereby augmenting T-cell-activation [4]. An association between CD4 and the T-cell receptor (TcR) has been demonstrated on the basis of cross-linking experiments [5] and an implication of the molecule in the transduction of intracellular signal has been recently proposed [6]. CD4 is also known to bind the envelope glycoprotein gp120 of the human immunodeficiency virus HIV-I [7], acting as a cellular receptor for HIV-1 [8] and HIV-2 [9]. Among the clinical features of the acquired immunodeficiency syndrome (AIDS), enteropathies like diarrhea, malabsorption, enteritis and proctitis are common [10–12]. Recently, Adachi et al. [13] demonstrated that the human colon adenocarcinoma cell line HT29 was susceptible to HIV infection. However, only a small fraction (up to 0.1%) of these cells could be directly infected with HIV, in agreement with a weak expression of CD4 mRNA [13]. Moreover the authors failed to detect the presence of CD4 proteins on the surface of HT29 cells [13].

Since it is well established that HT29 is a heterogenous cell line [14,15], we looked to study the expression of the CD4 antigen in a well-characterized cell clone (HT29-D4) derived from HT29 cells by limit dilution technique [16]. This clone represents a valuable model to study morphological and biochemical events associated with the process of enterocytic differentiation because it can be induced to differentiate by a simple alteration of the culture medium (i.e. the replacement of glucose by galactose) [17,18].

In this report, we demonstrate that: (i) CD4 proteins are synthesized by undifferentiated HT29-D4 cells, as demonstrated by radioimmunoprecipitation studies after metabolic or cell surface labeling; (ii) CD4 molecules are restricted to the basolateral membrane of differentiated HT29-D4 cells. Therefore this cell line would prove to be a useful model for in vitro infection of colonic epithelial cells by HIV.

2. MATERIALS AND METHODS

2.1. *Antibodies and reagents*

Monoclonal anti-CEA antibody MAC 601 was obtained from Biosys (Compiègne, France). Monocloncal anti-alkaline phosphatase antibody was obtained from Peninsula Laboratories (Belmont, CA). Monoclonal anti-villin (ID2C3), anti-HLA class I (B9.12.1), anti-CD4 (13B8.2 and B14) antibodies were from Immunotech (Marseille, France). Mouse IgG and rabbit anti-mouse IgG_1 were from Nordic Immunological Laboratories (Tilburg, The Netherlands). Bovine serum albumin (BSA), lactoperoxidase and Protein A-Sepharose were purchased from Sigma (St. Louis, MO). Ca^{2+}-free medium (S-MEM) was from Gibco (Grand Island, NY). All other cell culture

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media and supplements were from Eurobio (Paris, France). L-[^{35}S]Methionine and Na^{125}I were purchased from Amersham (Les Ulis, France). ^{125}I-Protein A was a generous gift of Dr G. Rougon (CNRS URA 202, Marseille, France).

2.2. *Cell culture*

HT29 cells were obtained from Dr A. Zweibaum (INSERM U178, Villejuif, France) and cloned in our laboratory [16]. HT29-D4 clonal cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose and 10% fetal bovine serum (FBS) (undifferentiated cells). To induce the differentiation of HT29-D4 cells, the glucose-containing medium was replaced by glucose-free DMEM supplemented with 5 mM galactose and 10% dialysed FBS. Thereafter the cells were subcultured in this medium and referred to as differentiated cells. Human acute lymphoblastic leukemia cells of the line CEM were routinely grown in RPMI 1640 supplemented with 10% FBS.

2.3. *Cell surface iodination*

HT29-D4 and CEM cells were radioiodinated in the presence of lactoperoxidase as previously described [19] according to Goding [20].

2.4. *Metabolic labeling*

HT29-D4 cells growing in 75 cm^2 flasks (5×10^7 cells) were incubated for 1 h at 37°C with 5 ml of methionine-free DMEM, after which L-[^{35}S]methionine was added to a final concentration of 200 μCi·ml^{-1}. After 3 h at 37°C, cells were washed twice with phosphate-buffered saline (PBS), pH 7.4.

2.5. *Immunoprecipitation and electrophoresis*

Cell surface or metabolically labeled cells were solubilized for 1 h at 4°C in the presence of buffer A (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride and 2 mM benzamidine). Lysates were precleared by centrifugation (10 min, 10000 × *g*). To reduce non-specific binding to the immunoadsorbent, cell lysates were also cleared by incubation with mouse IgG in the presence of Protein A-Sepharose at 4°C with shaking for 6 h. The mixture was centrifuged in an Eppendorf microcentrifuge for 2 min. Aliquots of the supernatant were incubated with the different monoclonal antibodies used in this study and Protein A-Sepharose at 4°C and shaken overnight. The monoclonal antibody 13B8.2 was insolubilized in the presence of rabbit anti-mouse IgG$_1$ polyclonal antibodies. Immunoprecipitates were washed 3 times with buffer A, then twice with the same buffer containing 500 mM NaCl, once again in buffer A and finally resuspended in electrophoresis sample buffer. The immunoprecipitated material was analysed by SDS-PAGE according to Laemmli [21]. The gel was dried and exposed to Fuji X-ray film with an intensifying screen (Du Pont).

2.6. *Binding studies*

Differentiated HT29-D4 cells were grown at confluency in 2 cm^2 well multidish plates in the presence of glucose-free, galactose-containing medium. Cell monolayers were rinsed twice in PBS 0.1% BSA and incubated for 2 h at 13°C with the indicated monoclonal antibody. Cells were then washed twice with PBS 0.1% BSA and incubated with ^{125}I-Protein A (10^5 cpm) for 1 h. After 3 washes in PBS 0.1% BSA, cells were lysed with NaOH (0.5 N). Radioactivity bound to the cells was determined in a γ-radiation spectrometer. When mentioned, junctional complexes were disrupted by incubating the cells in Ca^{2+}-free medium for 1 h at 37°C according to Godefroy et al. [22].

2.7. *Immunofluorescence studies*

Indirect immunofluorescence was performed as previously described [17].

3. RESULTS AND DISCUSSION

The ability of HT29-D4 cells to synthesize CD4 molecules was investigated by using radioimmunoprecipitation techniques. In a first set of experiments, the cells were incubated in the presence of L-[^{35}S]methionine, then lysed with detergent and immunoprecipitated with two different monoclonal antibodies directed against CD4 molecules, namely 13B8.2 and B14. As shown in the autoradiogram in Fig. 1, a major polypeptide with an apparent M_r of 60000 was specifically precipitated by these monoclonal antibodies (lanes 4 and 5). The other bands were presumably non-specifically precipitated because they were also observed after incubation of the labeled lysate with an irrelevant antibody (lane 2). An anti-HLA class I monoclonal antibody was used as a control, it immunoprecipitated two labeled polypeptides of 41 kDa and 12 kDa corresponding respectively to the HLA class I chain and the β_2-microglobulin associated polypeptide (lane 3). No labeled protein with an apparent M_r of 60000 was detected using this antibody.

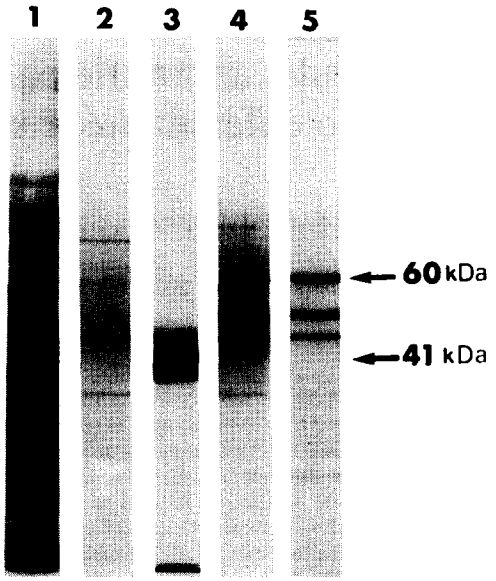

Fig. 1. Immunoprecipitation of metabolically labeled cell lysates. 5×10^7 HT29-D4 cells were labeled for 3 h with 200 μCi·ml^{-1} L-[^{35}S]methionine and the lysate (lane 1) was immunoprecipitated using 10 μg of control (lane 2), anti-HLA class I (lane 3), 13B8.2 (lane 4) or B14 (lane 5) in the presence of Protein A-Sepharose. Samples were then analyzed by SDS-PAGE and autoradiography.

In a second set of experiments HT29-D4 cell surface proteins were radioiodinated with Na^{125}I using the lactoperoxidase technique. After solubilization and immunoprecipitation with 13B8.2 and B14, a single polypeptide of M_r 60000 was specifically detected on the autoradiogram (Fig. 2A, lanes 3 and 4). The specificity of this immunoprecipitation reaction was assessed by using an irrelevant antibody (lane 1) or the anti-HLA class I antibody (lane 2) that immunoselected the HLA class I-β_2-microglobulin complex. In both cases, no detectable band was seen in the 60000 region of the gel. A similar experiment was performed on

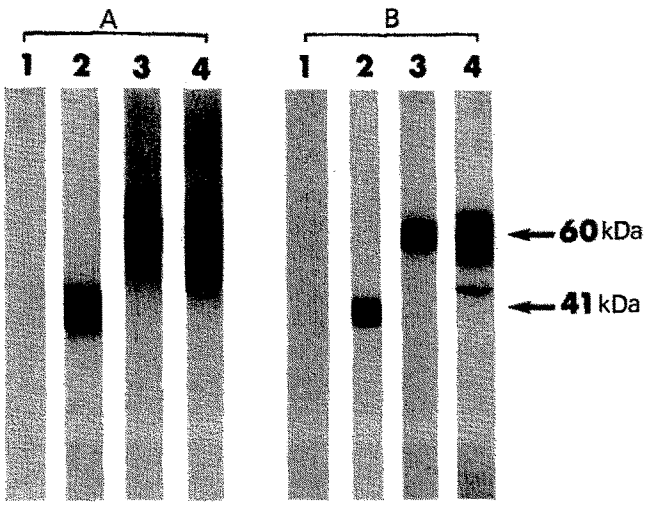

Fig. 2. Immunoprecipitation after cell surface labeling of HT29-D4 and CEM cells. HT29-D4 (A) and CEM (B) cells were radioiodinated using lactoperoxidase. After solubilization, the lysates were immunoprecipitated using 10 μg of control (lanes A1, B1), anti-HLA class I (lanes A2, B2), 13B8.2 (lanes A3, B3) or B14 (lanes A4, B4) in the presence of Protein A-Sepharose. Samples were then analyzed by SDS-PAGE and dried gels were autoradiographed at −80°C between intensifying screens.

human lymphoblastic cells CEM, which are known to express the CD4 antigen on their plasma membrane [23]. The results shown in Fig. 2B (lanes 3 and 4) demonstrate that a single polypeptide with an apparent M_r of 60000 was also specifically detected in these cells after immunoprecipitation with 13B8.2 and B14. Therefore, the 60 kDa polypeptide immunoprecipitated with 13B8.2 and B14 after both metabolic and cell surface labeling of HT29-D4 cells is likely the CD4 protein coded by the CD4 mRNA identified in HT29 cells by Adachi et al. [13].

The establishment of a differentiated state of the clonal HT29-D4 cells can be obtained by a simple change of the carbon source in the culture medium, namely the replacement of glucose by galactose [16]. When grown in glucose-free, galactose-containing medium, the cells form a polarized monolayer with numerous domes indicative of a functional epithelial differentiation [16,17]. At the structural level the cells exhibit a typical enterocytic differentiation characterized by an apical brush border facing the medium, with well organized microvilli and mature junctional complexes that divide the cell membrane in two independent domains, i.e. apical and basolateral [16,17,19]. These two domains differ strongly in their protein content as demonstrated by radioiodination and immunoprecipitation studies: sucrase-isomaltase, alkaline phosphatase and CEA are localized on the apical cell membrane whereas the VIP receptor, the transferrin receptor and the HLA class I antigen are restricted to the basolateral domain ([17,19] and in press).

The localization of CD4 molecules in dome-forming

Table I

Radioimmunoassay on differentiated HT29-D4 cells

Monoclonal antibody	cpm bound	
	Apical binding[a]	Total binding[b]
Anti-villin	140 ± 18	220 ± 36
Anti-alkaline phosphatase	11075 ± 510	10116 ± 1317
Anti-HLA class I	1931 ± 51	13914 ± 708
Anti-CD4*	1995 ± 107	7032 ± 339

After treatment with Ca^{2+}-containing ([a]) or Ca^{2+}-free ([b]) media, dome-forming HT29-D4 cells were incubated for 2 h at 13°C in the presence of the indicated antibody and in the case of monoclonal anti-villin antibodies with rabbit anti-mouse IgG_1. After two washes, ^{125}I-Protein A was added (10^5 cpm) for 1 h at 13°C. Radioactivity bound to cells was determined after lysing the cells in NaOH (0.5 N). Results are exposed as mean ± SD of 2 separate experiments performed in duplicate. * B14

HT29-D4 cells was investigated using a radioimmunoassay involving ^{125}I-Protein A as revealing agent. Specific apical binding was measured after incubation of HT29-D4 cells in Ca^{2+}-containing medium and compared with the total specific binding obtained after preincubation of cells in Ca^{2+}-free medium to open tight junctions [22]. Non-specific binding was measured using a monoclonal antibody directed against villin, a cytoskeletal protein. Results shown in Table I demonstrate that the binding of a monoclonal antibody recognizing an apical protein (e.g. alkaline phosphatase) is similar with or without treatment in Ca^{2+}-free medium. On the contrary, a basolateral marker (e.g. HLA class I antigen) is not accessible to a specific monoclonal antibody because tight junctions prevent the antibody access to the basolateral side of the cells. In Ca^{2+}-free medium, these junctions open and a free access is allowed to basolateral proteins. Thus, binding of anti-HLA class I antibodies is much more important when done on HT29-D4 cells preincubated in a medium devoid of Ca^{2+}. This result is in agreement with the known basolateral restriction of HLA class I molecules in these cells [17,22] as well as in normal intestinal epithelium [24].

The binding of anti-CD4 monoclonal antibody B14 is also strongly enhanced after opening tight junctions, indicating that most of CD4 molecules were restricted to the basolateral domain of polarized HT29-D4 cells. It should be noted that the failure to detect any significant binding of anti-villin antibodies rules out the possibility of an eventual toxicity of the Ca^{2+}-free medium that could have led to a free access of anti-CD4 antibodies to intracellular rather than basolateral proteins.

We also performed indirect immunofluorescence using the anti-CD4 antibody to visualize the distribution of CD4 molecules at the surface of differentiated HT29-D4 cells. As control antibodies, anti-CEA and anti-HLA class I were also tested. These data are shown

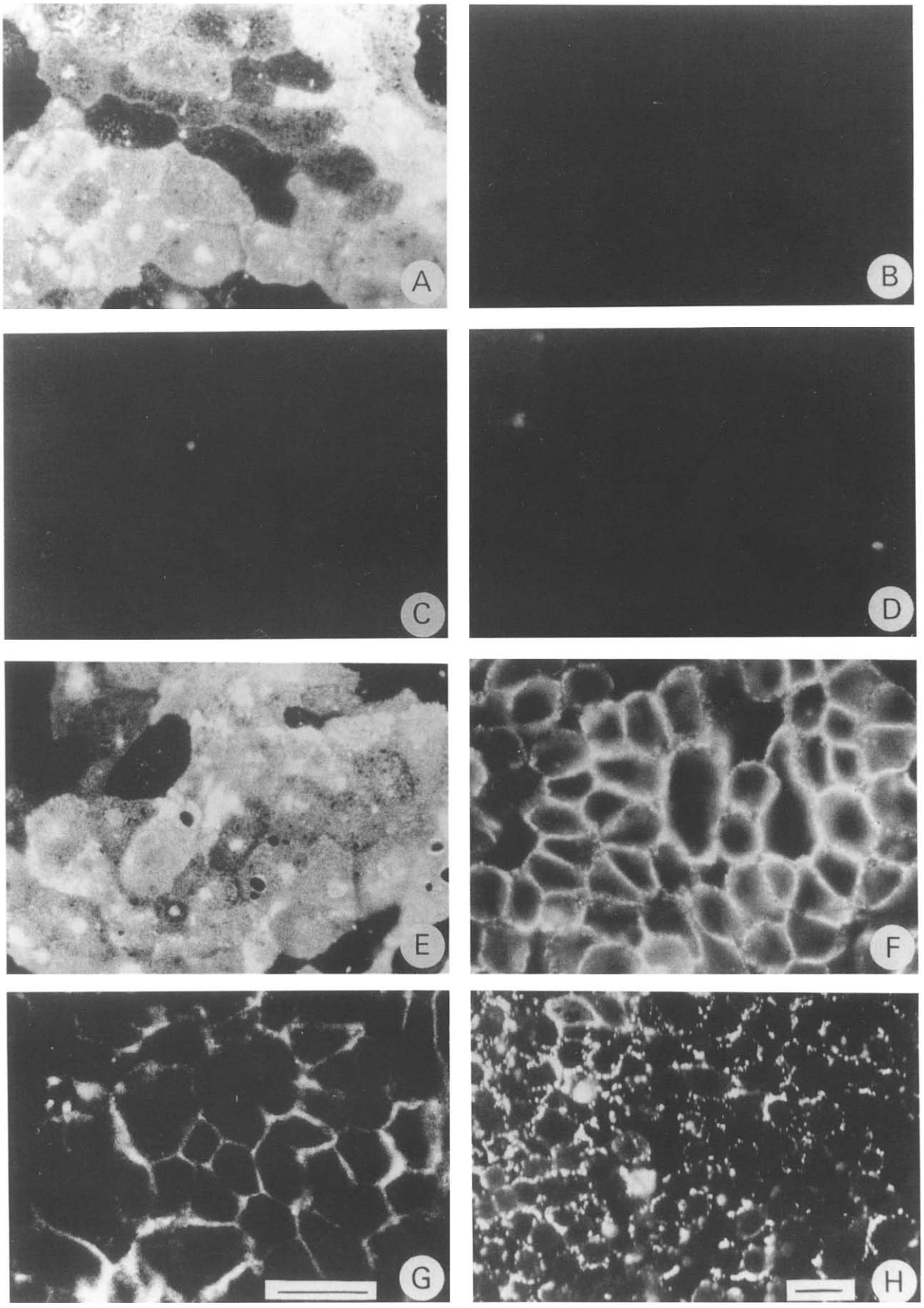
A
B
C
D
E
F
G
H

Fig. 3. Immunofluorescent staining of differentiated HT29-D4 cells. HT29-D4 cells were induced to differentiate in glucose-free, galactose-containing medium and cultured on glass coverslips until dome formation was observed in post-confluent monolayers. Cells were preincubated in Ca^{2+}-containing (A–D) or Ca^{2+}-free (E–H) medium and immunolabeled using anti-CEA (A, E), anti-HLA (B, F), 13B8.2 (C, G) or B14 (D, H) and FITC rabbit anti-mouse IgG. When pretreated with Ca^{2+}-containing medium, HT29-D4 cells were stained only with the anti-CEA monoclonal antibody (A) since the tight junctions prevented the access of anti-HLA (B), 13B8.2 (C) and B14 (D) to the basolateral membrane. After treatment in Ca^{2+}-free medium, tight junctions were opened and basolateral membrane were uniformly stained with anti-HLA (F), 13B8.2 (G) and B14 (H). The labeling with anti-CEA remained apical even after the junctions were disrupted (E). Bars: 20 μm (A–G); 50 μm (H).

in Fig. 3. When the experiment was performed on intact dome-forming HT29-D4 cells, using an anti-CEA monoclonal antibody, a typical punctated fluorescence, characteristic of an apical labeling viewed en face (Fig. 3A). On the contrary, no detectable fluorescence was obtained when anti-HLA class I antibodies were used unless the junctional complexes were disrupted in the presence of Ca^{2+}-free medium for 1 h at 37°C (Fig. 3B and F). The membrane distribution of CD4 molecules behaved like HLA class I molecules because the fluorescent labeling with the two monoclonal antibodies directed against CD4 (i.e. 13B8.2 and B14) was detected exclusively on Ca^{2+}-free medium pretreated cells (Fig. 3G and H), in agreement with the data we obtained using radioimmunoassay (Table I). All these data are consistent with a basolateral localization of CD4 molecules at the surface of polarized HT29-D4 cells.

Because HT29-D4 cells can be readily grown on permeable filters where they form leakproof electrically active monolayers [17,19,25], we will take advantage of this culture method that gives independent access to each domain of the cell membrane to try to selectively infect HT29-D4 cells with HIV by the basolateral membrane domain which contains CD4 molecules (as demonstrated in the paper) or by the apical domain as a control infection. The polarized expression of virus envelope proteins [26] will be studied in this model. In conclusion, the HT29-D4 clone will undoubtedly be a useful model to study the infection of colonic epithelial cells by HIV and to help understand more precisely some of the gastrointestinal disorders described in AIDS patients [10–12].

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