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A simple assay based on HIV infection preventing the reclustering of MT-4 cells*

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This report confirms and extends the recent work of Pauwels et al. on a "reclustering" assay (a simple microtitration plate method) for the determination of human immunodeficiency virus (HIV) infection of MT-4 cells. MT-4 cells, which are highly susceptible to and permissive for HIV, typically grow in clusters. In the absence of virus these cell aggregates, after dissociation by pipetting, reform into clusters within 2 to 3 hours. Growth of HIV results in an inhibition of reclustering, with an end-point some 4–5 days after initiation of infection. In cultures inoculated with 5 to 8 $TCID_{50}$ of HIV, only 2–4% of the cells remain viable after 4 days. Correspondingly, HIV antigens can be detected by immunofluorescence in more than 90% of the cells remaining in the culture. The sensitivity of the "reclustering" assay is only slightly less than that of the immunofluorescence test. A colorimetric assay is also described that employs a tetrazolium salt (designated as MTT) to measure the cytolytic effect of various dilutions of HIV; comparable virus titres were obtained. This reclustering assay now appears to offer the simplest method for titration of prototype HIV in virus stocks and when used in drug evaluation tests and for measurement of HIV neutralizing antibodies.

Since the discovery of the human immunodeficiency virus (HIV) (*1*, *2*, *6*) much effort has gone into finding susceptible, highly permissive cell lines and developing reliable assays for detecting multiplication of the virus. The first isolations of HIV were made from mononuclear cells in the peripheral blood of patients with acquired immunodeficiency syndrome (AIDS) and the first successful transmission of HIV to an established T-cell line, H9, was achieved by Popovic et al. (*10*). Other T-cell lines were found to be susceptible to HIV infection. Each of these cell lines had one thing in common—the presence of a CD4 epitope on the surface of the cell to which the envelope protein of HIV could bind. Subsequently, it was reported that HIV could replicate in circulating monocytes, macrophages, and glial cells and in Epstein-Barr virus-transformed B lymphoblastoid cell lines. However, the incubation period is relatively long in these cell lines, with the cells manifesting partial resistance to the cytopathic effect of the virus.

Recently, Harada et al. (*3*) reported that a lymphocyte cell line, MT-4, which carries the HTLV-I genome was highly susceptible to HIV infection. After superinfection with HIV, a rapid induction and release of HIV antigens were observed accompanied by a marked cytopathogenic effect (*4*). This feature allowed Harada et al. (*3*) to successfully develop a

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plaque neutralization assay. The assay was based on an inhibition of the cytopathic effect on MT-4 cells after HIV superinfection (*5, 11*). In 1987, Pauwels et al. (*9*) described a simple, rapid, and sensitive microassay based on the dissociation and reclustering of MT-4 cells. Clusters of uninfected cells, when dissociated by pipetting, reclustered after 3 hours of incubation at 37 °C, but reclustering was not observed if the cells had been infected with HIV.

We have further characterized the cluster formation and reclustering of uninfected and HTLV-III$_B$-infected MT-4 cells. In a microplate assay system, we have measured the speed of reclustering, the influence of viability and age of the MT-4 cells on the formation of clusters, and the inhibitory effect of HIV infection on reclustering using trypan blue dye exclusion, indirect immunofluorescence, a colorimetric assay in which a tetrazolium salt is used to detect living cells (*8*), and electron microscopy. The reclustering procedure allows a rapid evaluation of infection in MT-4 cells with the established HTLV-III$_B$ stock virus. In addition, the tetrazolium salt procedure may be an important addition to simplifying the assay of HIV.

MATERIALS AND METHODS

Cells

The MT-4 cell line, carrying human T-lymphotropic virus type I (HTLV-I), was established by Miyoshi et al. (*7*) and was kindly provided by Dr J. Gerin (Georgetown University School of Medicine, Rockville, MD). The cells were grown in 25-cm^2 cell-culture flasks with RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco). The cells were subcultured in fresh medium at a concentration of 5×10^5 cells/ml every 3–4 days. The cell count and viability were measured by a trypan blue dye exclusion technique.

Virus preparation

HIV was prepared from the supernatant of the H9/HTLV-III$_B$-infected cell line which was kindly provided by Dr M. Popovic (National Cancer Institute, Bethesda, MD). HIV-infected H9 cells were grown in 75-cm^2 culture flasks in RPMI 1640 medium with 15% heat-inactivated FBS. For virus purification, supernatant fluids from several flasks were pooled and then clarified by centrifugation at 10 000$\times g$ for 20 min at 4 °C. The clarified culture fluid was recentrifuged at 150 000$\times g$ for 45 min at 4 °C to pellet the virus. Virus pellets were resuspended in RPMI 1640 medium containing 20% FBS at one-tenth the original volume and stored at −70 °C in 0.5-ml aliquots. The HIV stock preparation contained 3.4×10^9 physical particles per ml, as determined by electron microscopy, and had a $TCID_{50}$ titre of $10^{3.8}$ titrated in H9 cells using an enzyme immunoassay HIV p24 antigen detection system (DuPont, Boston, MA) to determine viral replication.

Human anti-HIV sera

Sera with high titres of antibodies against HIV (anti-HIV) were selected from our serum bank of haemophiliacs. They were tested by enzyme-linked immunosorbent assay (ELISA) HTLV-III (Abbott) and by indirect immunofluorescence using HIV-infected H9, MT-4, and THP-1 cells. No staining of uninfected cells was observed with these sera.

Reclustering assay with uninfected and HIV-infected MT-4 cells

The procedure followed that described by Pauwels et al. (*9*). Briefly, MT-4 cells that had been subcultured 24 hours previously were suspended in 15-ml sterile plastic centrifuge tubes at a concentration of 5×10^5 cells/ml. The cells were washed twice with RPMI 1640 medium, following which the cell pellets were infected with 200 µl of diluted HIV stock preparation. Virus dilutions were prepared in RPMI 1640 medium; this medium was also used for mock infection of MT-4 cells. Cell-virus mixtures were incubated at 37 °C for 1.5 h, after which 1.8 ml of RPMI 1640 medium with 10% FBS was added, and 100 µl of the suspended cells was added to the wells of a flat-bottom, 96-well plastic microtitration plate (Falcon-3872, Becton Dickinson; Labware, Oxnard, CA). The cell cultures were incubated at 37 °C in a 5% CO_2 and 95% air humidified incubator. On specified days, clusters of MT-4 cells were dissociated by pipetting (8–10 strokes with a 50 µl octapipette) and then allowed to recluster at 37 °C. Experiments were also carried out to evaluate the reclustering at different time periods using normal uninfected MT-4 cells. Clusters were monitored microscopically.

Indirect immunofluorescence test for HIV antigen expression

On different days, the infected and uninfected MT-4 cells in the microtitration plate wells were resuspended in the culture medium and transferred to 1.5-ml plastic centrifuge tubes. After pelleting the cells (1 min, Fisher Micro-Centrifuge model 59A) and washing them once with 1 ml of phosphate-buffered solution (PBS) (pH 7.2), the cell pellets were resuspended in 10 µl of PBS and 5 µl of each sample was placed onto microscope slides. The cells

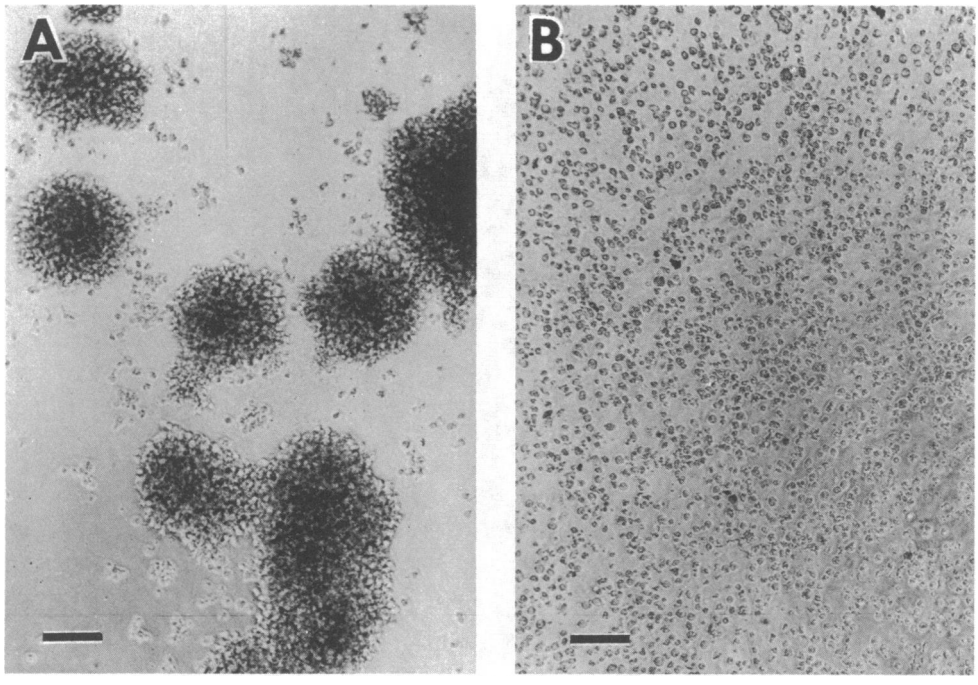


Plate 1. Phase-contrast microscope pictures of MT-4 cells after mock infection (A) and HIV infection (B) (approx. 40 $TCID_{50}$ per well) at 5 days postinfection. Cells were incubated for 2 hours at 37 °C after dissociating them by pipetting. The mock-infected cells reformed clusters (A), but the HIV-infected cells (B) did not recluster (bar = 100 μm).

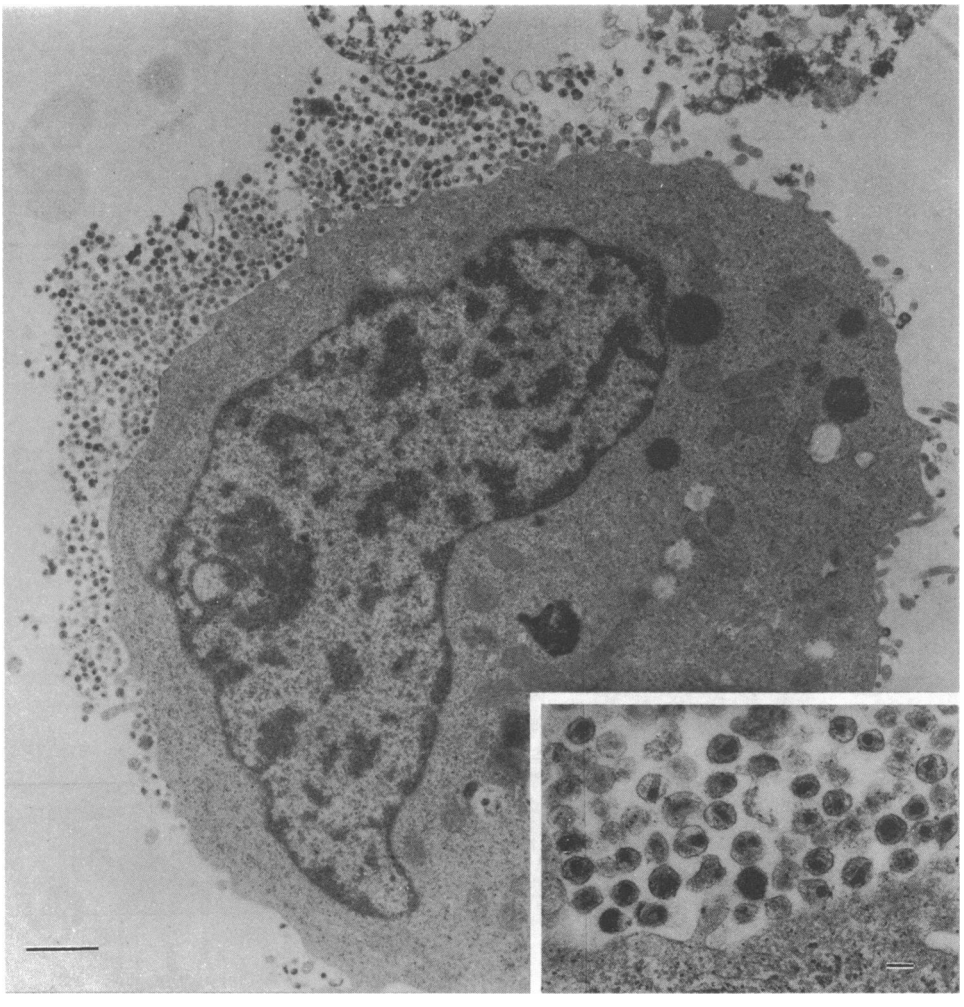


Plate 2. Electron micrograph of an MT-4 cell infected with HIV. At 5 days postinfection, evenly dispersed nucleoplasm and disrupted cellular organelles with vacuoles and lipid droplets can be seen. Many viral particles are present extracellularly at or near the cytoplasmic membrane (bar = 1 μm). Insert shows typical HIV particles with cylindrical nucleoid or condensed eccentric cores (bar = 100 nm).

were air-dried and then fixed in cold acetone at −20 °C for 15 min. The fixed and dried MT-4 cells were either tested immediately or stored in absorbent paper and aluminium foil at −70 °C until use.

For immunofluorescent staining, 10 μl of HIV-positive serum (previously inactivated at 56 °C for 30 min and diluted in PBS) was placed over each cell-spot. After incubation for 45 min at 37 °C in a humidified chamber, the slides were washed twice with PBS for 10 min and then incubated, as previously described, with IgG goat-antihuman polyvalent serum conjugated with fluorescein isothiocyanate (Sigma No. F-6506, diluted 1:30 in PBS). The slides were rewashed with PBS, rinsed in distilled water and, after drying, mounted in a water-soluble medium (Elvanol) prior to observing for specific staining on a Zeiss epifluorescence microscope. HIV-negative sera, uninfected MT-4 cells, and conjugated goat-antihuman serum alone were used as negative controls in all tests.

Assay for cytotoxic effect of HIV using a tetrazolium salt

A colorimetric assay for detecting living cells (*8*) was adapted to our studies. The method utilizes the tetrazolium salt, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), which is a pale-yellow substrate until it is metabolically changed to dark purple by the presence of active mitochondria in living cells. Briefly, 10 μl of a freshly prepared MTT stock solution (Sigma No. M2128, 5 mg per ml in PBS) was added to each well of MT-4 cells in 100 μl of culture fluid. After 90 min incubation at 37 °C, the medium was carefully removed by a micropipette or preferably by a 0.5-ml glass syringe using a 26-gauge, 3/8 inch needle (Becton-Dickinson No. 5260) to avoid inadvertent loss of cells. The dark-purple MTT-formazan product was solubilized by the addition of 100 μl of dimethyl sulfoxide (Sigma No. D5879), following which the intensity of colour was read at 640 nm with a plate-reader (DuPont Microplate Reader) and the optical density was compared to control values. More than 50% inhibition of formazan production was accepted as positive, e.g., as indicating significant cell death. To improve precision of the assay, samples were tested in triplicate.

Electron microscopy of HIV

For negative staining of free HIV, the supernatants of HIV-infected MT-4 cell cultures were clarified at 12 000×*g* for 15 min at 4 °C. Samples were diluted 1:2 in 3% glutaraldehyde (EM grade; Electron Microscopy Sciences, Fort Washington, PA) to inactivate the virus and then the particles were stained with 1% ammonium molybdate.

Cell samples also were prepared for investigation. The cells were washed twice with PBS and then fixed in 3% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in increasing concentrations of ethanol, and embedded in Epon-Araldite. Ultrathin sections were cut and poststained with 2% aqueous uranyl acetate followed by lead citrate. They were examined with a Philips SM-10 transmission electron microscope at 60 kV.

RESULTS

Growth pattern and viability of MT-4 cells

MT-4 cells, after being seeded in the microtitration plate wells, grew in typical clusters or clumps (Plate 1A). When about 5×10^4 uninfected cells were placed in a well, 10–15 individual clusters of cells could be detected. Individual cells were not completely rounded but had irregular contours. There was no fusion between cells, and the clusters were easily dissociated by pipetting. The viability of cells subcultured 24 hours previously was high (90–95%), began to decline after 48 hours in culture, and showed a major decline between days 4 and 5 when only half the cells remained viable (Fig. 1).

Speed of the reclustering

When the 24-hour-old uninfected MT-4 cells were

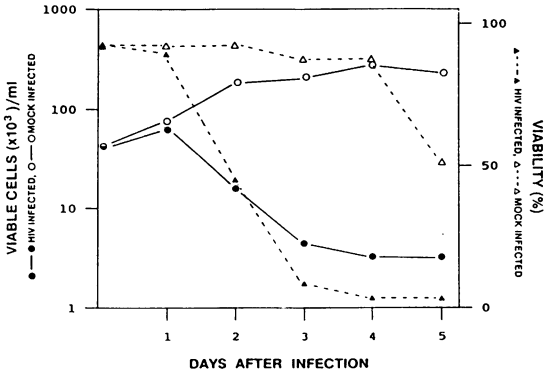


Fig. 1. Numbers and viability of MT-4 cells after mock infection and HIV infection. The MT-4 cells (24 hours old) were grown in flat-bottom, 96-well microtitration plates and were infected with HIV (approx. 40 $TCID_{50}$ per well) or mock infected. Triplicate samples of HIV- and mock-infected cells were counted each day in a haematocytometer using the trypan blue dye exclusion method and the counts were averaged.

added to the wells of microtitration plates and observed 24 hours later, almost all the cells formed clusters. After dissociation of the clusters, the cells began reclustering after 10 min (Table 1). The number of cells in clusters increased rapidly and, after 90 min incubation, there were too many cells to count. At 3 hours, very few live single cells remained. A slight increase in speed of reclustering was evident as the age of the cells in culture increased, i.e., older cells formed clusters more rapidly.

Effect of HIV on the viability and morphology of MT-4 cells

MT-4 cells inoculated with HIV (about 40 $TCID_{50}$ per well) showed a markedly different cell growth and viability pattern when compared with mock-infected cells (Fig. 1). The number of viable cells rapidly decreased at 24 hours postinfection, and by day 4 only 2–4% of the infected MT-4 cells were viable. In contrast, the mock-infected cells grew well, reaching a plateau between days 2 and 4. After 4 days, the viability of these cells began to decline appreciably. For both infected and uninfected MT-4 cells, viable cells were found mainly in the clusters. The infected cells became round, lost their surface characteristics, became refractile and diminished in size. By day 3, many infected cells developed a balloon-like, cytoplasmic swelling, a morphological observation which later disappeared. Some multinucleated giant cells could be observed among the remaining 2–4% live cells. The dose of virus influenced the number of viable cells and the time course of appearance of these cytopathic effects (data not shown).

Detection of HIV infection by reclustering and immunofluorescence assay

The propagation of HIV correlated well with the occurrence and degree of cytotoxicity, the detection of HIV p24 antigen in the culture supernatant, and the appearance of viral antigen in the cells. By the third day, dissociation of the clusters of infected cells by pipetting was followed by partial reclustering. Over the next two days the cells completely lost their ability to recluster (Plate 1B) while the uninfected control cells continued to form clusters within 2 hours. After 5 days, the substantial increase in the number of dead cells observed in the mock-infected cultures made it difficult to evaluate the assay.

Expression of viral antigens in HIV-infected MT-4 cells was very rapid. Nearly 50% of the cells showed specific fluorescence after 48 hours in culture (Table 2). The percentage of positive cells continued to increase and exceeded 90% by day 4. Later, most of the cells became pyknotic, but retained their bright fluorescence. No fluorescent cells were observed in the uninfected MT-4 control cells. HIV infection was detected by the reclustering assay two days after the indirect immunofluorescence antibody (IFA) test became positive. When more than 80% of the cells were IFA-positive, the "reclustering" assay showed a clear difference between infected and uninfected cultures. When the cultures were re-examined 24 hours after pipetting, no reclustering had occurred in the infected cultures.

Determination of the cytotoxic effect of HIV in MT-4 cells using the MTT colorimetric assay

MT-4 cells in 4-day-old cultures (about 5×10^4

Table 1. Speed of reclustering of uninfected MT-4 cells on different days in culture after dispersion of cell clusters by pipetting; cells were cultured in microtitration wells (5×10^4 cells/100 μl medium/well) and were resuspended by pipetting at the indicated time intervals; reclustering was monitored continuously by microscopic observation during the first 20 min; after 20 min, the plate was incubated at 37 °C between observations

Culture age (hours)[a]	Time after dissociation of clusters (min)							
	5	10	20	30	60	90	120	180
24	—[b]	±[b]	+[b]	+	+	++[b]	++	+++[b]
48	—	±	+	+	++	++	++	ND[c]
72	—	±	+	++	++	++	+++	+++
96	—	±	+	++	++	++	ND[c]	+++
120	—	±	+	++	++	++	+++[d]	+++[d]

[a] In microtitration wells.

[b] The numbers of cells making up the clusters were counted and quantified as follows: —, no reclustering; ±, reclustering with <20 cells per cluster; +, reclustering with >20 cells per cluster; ++, reclustering with too many cells to count per cluster; +++, reclustering similar to controls.

[c] Not determined.

[d] Many dead single cells were seen among the live clustered cells.

Table 2. HIV infection detected by reclustering assay and immunofluorescence (IF) test; mock- and HIV-infected (40 $TCID_{50}$ per well) MT-4 cells were dissociated by pipetting on different days and the reclustering was monitored 2 hours later; cells were harvested in parallel for IF staining using HIV antibody-positive human serum

Culture age (hours)	Reclustering assay		Immunofluorescent test	
	HIV-infected MT-4 cells	Mock-infected MT-4 cells	HIV-infected MT-4 cells	Mock-infected MT-4 cells
24	+[a]	+	ND[b]	ND
48	+	+	+ (50)[c]	–[c]
72	±[a]	+	+ (80)	–
96	–[a]	+	+ (>90)	–
120	–	+	+ (>90)	–
144	–	±[d]	ND	ND

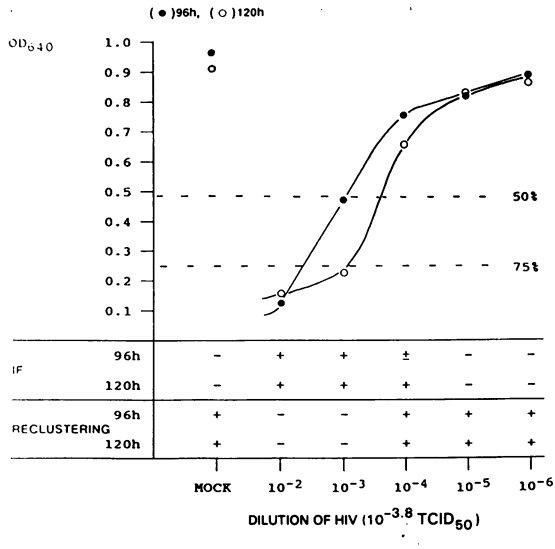
[a] Complete (+), partial (±), or no (–) reclustering was observed.
[b] Not determined.
[c] Specific IF staining (+ for positive and – for negative) with percentage of positive cells in parentheses.
[d] Many dead single cells remained among the live clustered cells.

cells/well) incorporated enough MTT during a 90-min incubation period at 37 °C to give an optical density of about 1.0 at 640 nm wavelength. HIV-infected cells showed a decreased ability to change the tetrazolium salt to formazan, depending on the dose of virus inoculated (Fig. 2). At 4 days post-infection, the inhibition of formazan production exceeded 50% of the uninfected control value in cultures infected with about 8 $TCID_{50}$ virus dose, and reached 75% by day 5.

The reclustering assay showed the cytotoxic effects of HIV at the same virus dilution but not at a dilution of 10^{-4}. The immunofluorescence assay was slightly more sensitive, revealing HIV-induced antigens by day 5 in cells harvested from triplicate cultures infected with a virus dose of about 1.0 $TCID_{50}$.

Electron microscopy

Supernatants of 5-day-old microtitration cultures contained about 10^9 physical particles per ml by negative staining, and the infected MT-4 cells after embedding and sectioning also showed a high level of virus production (Plate 2). Budding could be observed; however, the majority of virus particles were present extracellularly at or close to the cytoplasmic membranes. At 3 days postinfection, the virus particles which were 70–130 nm in diameter and had a cylindrical nucleoid or a condensed circular, eccentric core, were found near the cell membrane. The cells exhibited irregularly indented or lobulated nuclei, evenly dispersed nucleoplasm, and disruption of the cellular organelles with vacuoles, lysosomal granules, and lipid droplets. Cytoplasmic fusions and giant-cell formation were not prominent.



* MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)

Fig. 2. Cytotoxic effect of HIV on MT-4 cells using MTT staining. The cytotoxic effect of HIV is expressed as an inhibition of formazan production by the MT-4 cells. Results are compared with those of reclustering and immunofluorescence (IF) tests. 10 µl of MTT stock solution (5 mg/ml in PBS) was added to 100 µl of culture fluid per well. After incubating the reactants for 90 min at 37 °C, the medium was discarded. Formazan was solubilized by the addition of 100 µl of dimethylsulfoxide, and the colour intensity was measured photometrically at 640 nm. Results are the average of three replicates.

DISCUSSION

The HTLV-I transformed cell line, MT-4, with its reclustering properties was found to be a valuable tool in microassay for monitoring HIV infection within 4–5 days. In our experiments, MT-4 cells were easily propagated without interleukin-2, phytohaemagglutinin, or other expensive ingredients. Furthermore, they did not require pretreatment, e.g., Polybrene or DEAE-dextran, for infection by HIV, at least when the prototype HTLV-III$_B$ strain was used.

The MT-4 cells grow in clumps or clusters that can be disrupted easily by simple pipetting. As used here, they do not appear to participate in cell fusion or syncytium formation to any significant extent. The dissociated cells reclustered faster in 3–4-day-old clusters than they did after 24 hours. The reclustering property of MT-4 cells, and its inhibition by HIV infection, allowed the development of a sensitive and rapid microassay. In uninfected cultures, the reclustering begins early after the dissociation of clusters by pipetting, and 60–90 min later the majority of cells have reformed into clumps (see Plate 1A). Prototype HIV, even at a low dose (e.g., 8 TCID$_{50}$), can completely inhibit the reclustering of infected cells at 4 or 5 days postinfection (Plate 1B). By this time, only a few live single cells remain, some of which are seen forming multinucleated giant cells, and HIV antigens are detected in more than 90% of cells. The number of free virus particles detected by electron microscopy was near 10^9 per ml.

Parallel experiments indicated that the sensitivity of the reclustering assay was less than one log below the end-point titre observed using the indirect immunofluorescence assay when the cultures were evaluated at 4 or 5 days postinfection. When approximately 80% of cells contained viral antigens, the number of cells remaining had decreased to less than 4% of the original number added to the culture, which is in good agreement with the published data (*3*, *9*). When the number of viable cells exceeded 20–25%, the assessment of the reclustering assay was difficult because the live cells formed clumps among the dead cells.

Because the MT-4 cells are exquisitely sensitive to the cytotoxic effects of HTLV-III$_B$, they are ideally suited to evaluation with a metabolic assay using MTT as a measure of cell viability. However, this procedure was less effective using other cell lines (e.g., H9, Sup-T1 or THP-1 cells) because these cell lines become permanently infected and do not show the marked cytotoxic effects seen with MT-4 cells (unpublished observation). The method is suitable for development as a rapid screening method for a large number of samples.

In summary, we have studied the cluster formation that occurs during the culture of MT-4 cells and its abolition following HIV infection with HTLV-III$_B$. The results show that MT-4 cells produce virus-specific antigens and progeny virus within 4–5 days, accompanied by a significant cytolytic effect. These events permit one to monitor and quantify HIV in a very simple reclustering assay. The results observed are comparable to the immunofluorescence test and a metabolic staining method. Consequently, the reclustering assay is suitable for experiments where prototype HIV with MT-4 cells can be used, e.g., titration of prototype HIV when used in drug evaluation tests and measurement of HIV neutralizing antibodies.

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RÉSUMÉ

UNE ÉPREUVE SIMPLE FONDÉE SUR LE FAIT QUE L'INFECTION À VIH EMPÊCHE LA RÉAGRÉGATION DES CELLULES MT-4

Ce rapport confirme et complète les travaux récents de Pauwels et al. sur une méthode de "réagrégation" (une méthode simple de microtitrage) permettant de déterminer l'infection des cellules MT-4 par le virus de l'immunodéficience humaine (VIH). Les cellules MT-4, qui sont extrêmement sensibles et permissives au VIH, se multiplient généralement en îlots. En l'absence de virus, ces agrégats de cellules, après avoir été dissociés par aspirations-refoulements successifs, se reforment en îlots dans les 2 à 3 heures. La réplication du VIH se traduit par une inhibition

de la réagrégation, le point final étant de 4 à 5 jours après le début de l'infection. Dans les cultures dans lesquelles on a inoculé 5 à 8 $DICT_{50}$ de VIH, seules 2 à 4% des cellules restent viables au bout de 4 jours. Les antigènes du VIH peuvent alors être décelés par immunofluorescence dans plus de 90% des cellules restant en culture. La sensibilité de cette méthode de "réagrégation" n'est que légèrement inférieure à celle de l'épreuve d'immunofluorescence. Est également décrite dans cet article une méthode colorimétrique faisant appel à un sel de tétrazolium (désigné sous l'abréviation MTT) pour mesurer l'effet cytolytique de diverses dilutions de VIH; des titres de virus comparables ont été obtenus ainsi. Cette méthode de réagrégation semble désormais offrir le moyen le plus simple pour titrer le virus prototype dans les préparations virales, lors de l'évaluation des médicaments, ainsi que pour le titrage des anticorps neutralisants anti-VIH.

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