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McCOY CELL LINE AS A POSSIBLE MODEL CONTAINING CD4+ RECEPTORS FOR THE STUDY OF HIV-1 REPLICATION

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SUMMARY

Several studies have recently shown the use of recombinant rabies virus as potential vector-viral vaccine for HIV-1. The sequence homology between gp 120 and rabies virus glycoprotein has been reported. The McCoy cell line has therefore been used to show CD4+ or CD4+ like receptors.

Samples of HIV-1 were isolated, when plasma of HIV-1 positive patients was inoculated in the McCoy cell line. The virus infection was then studied during successive virus passages. The proteins released in the extra cellular medium were checked for protein activity, by exposure to SDS Electrophoresis and blotting to nitro-cellulose filter, then reacting with sera of HIV positive and negative patients.

Successive passages were performed, and showed viral replication, membrane permeabilization, the syncytium formation, and the cellular lysis (cytopathic effect).

Flow cytometry analysis shows clear evidence that CD4+ receptors are present in this cell line, which enhances the likelihood of easy isolation and replication of HIV.

The results observed allow the use of this cell line as a possible model for isolating HIV, as well as for carrying out studies of the dynamics of viral infection in several situations, including exposure to drugs in pharmacological studies, and possibly studies and analyses of the immune response in vaccine therapies.

KEYWORDS: HIV-1; Viral Replication; Rabies virus; CD4+; McCoy cell line.

INTRODUCTION

Many studies have demonstrated details of molecular interaction between acquired immunodeficiency virus (AIDS/HIV) and the biology of host cells[14].

For the virus to enter a target cell, the viral envelope must fuse with the cell membrane. This fusion is mediated by the glycoprotein[48] of the viral envelope[10,21,54].

Selective pressure has imposed the need for specialization of viral proteins to permit the occurrence of interaction of the genetic material of the virus with the host cell[54].

HIV, in particular, uses the glycoprotein of 120 kDa molecular weight (gp120) of the viral envelope to adhere to the host cell. This adhesion depends on a specific receptor located on the cell membrane denoted CD4, which is also a glycoprotein of 55 kDa molecular weight. The interaction between the two molecules is of a high-affinity type, permitting the gp120 molecule and the surface receptor to perform endocytosis of the viral particle[11,21,54]. This mechanism has been preliminarily reported for target cells, i.e., T/CD4+ lymphocytes[16,38,51]. However, other cells also express the CD4 receptor, i.e., monocytes, dendritic cells, as well as glial cells of the central nervous system, in addition to macrophages, which are the main target cell (*in vitro)* for simian immunodeficiency virus (SIV)[54].

The main function of CD4 binding is to induce conformational changes in gp120 that contribute to the formation or expression of binding sites for cytochemical receptors[8,12,15,21,38]. Some HIV-1 and HIV-2 viruses isolated from culture cells in the laboratory, as well as many primary virus isolates - SIV - have proved not to depend on CD4 to enter the cell,

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but to be able to bind to chemokine receptors of the alpha and beta type (X4 and R5), act as co-receptors for HIV together with CD4, and carry out important functions in the isolation of some retrovirus types[4,15].

More recent studies have demonstrated that the CCR5 and CXCR4 co-receptors are related to membrane fusion when the virus enters the cell. The probable sequence of events is as follows: HIV first enters the cell, with the viral envelope binding to CD4 through gp120/41, a conformational change occurs in gp120, which is altered, thus binding to the co-receptors, and finally coalescence of the viral envelope and cell membranes occurs. Infected cells continue to express the gp120/41 receptor on the cell surface during infection both before the incorporation of newly formed virions (virus particles) by the cell membrane and after virion exocytosis (budding) into the extracellular medium[44,54].

Other studies have demonstrated the possibility that HIV pseudotypes may enter the cells through this same receptor[43,44] or closely similar receptors. These pseudotypes may be viruses like vesicular stomatitis virus (VSV), which is a rhabdovirus, that may be modified and induced to express proteins of the HIV-1 virus[30,42] . Anti-HIV vaccines have been based on these hybrid pseudotypes mimicking HIV, and have been developed with the VSV[42].The physicochemical and biological properties of these pseudoviruses have served as the basis for the production of anti-HIV vaccines[44].

Previous studies have also reported the homology of a protein sequence of HIV gp120 with the neurotoxic loop region of the rabies virus and also of the neurotoxins of snake venom[23,32,47]. The McCoy cell line was shown to be efficient for the replication and isolation of rabies virus[33,34].

The present investigation was based on the cited studies in order to evaluate HIV replication in McCoy cells and to test the hypothesis that McCoy cells have CD4 type or CD4-like receptors on the cell membrane.

MATERIAL AND METHODS

Cell culture*:* The McCoy lineage cells used were obtained from the Cell Culture Section of the Adolfo Lutz Institute, which is a hybrid lineage with markers from human cells and mouse cells. This cell is registered in the American Type Culture Collection under number CRL 1696[2].

Identification of HIV-1*:* The plasma of a patient infected with HIV-1 admitted to the Children's Hospital (São Paulo, Brazil) in 1994 was provided by the plasma bank, Section of Immunology, Adolfo Lutz Institute. The plasma (1 mL) was inoculated into cell culture plates 6 cm in diameter containing 3 ml Eagle medium supplemented with 5% fetal calf serum and a monolayer of confluent McCoy cells. Changes in cell morphology were observed until the occurrence of a total or partial cytopathic effect depending on viral titer. A one-mL sample was withdrawn after every passage, and once again inoculated into another bottle containing a monolayer of freshly split cells. Each successive passage of the inoculate remained until there was cellular lysis of the majority of cells, approximately 80% of cells. Duration ranged from 10 days in the first passages to 5 days in the last passages, when the titer was higher. Up to 11 passages were carried out.

Viral replication kinetics was performed and observed through changes in cell morphology; 0.2 mL of the sample of HIV-1 isolate in McCoy cells (5th passage) was reinoculated in a monolayer of cells for 30 minutes for adsorption of the virus in the cells and then a further 2 mL of Eagle medium supplemented with 2% fetal calf serum was added. Five more plates containing uninfected McCoy cells served as controls. All plates were observed for morphology for 120 hours; each pair of plates contained coverslips where the cells grew. Every 24 hours the coverslips (containing both infected and control cells) were fixed using an aqueous solution of alcohol and acetic acid and processed for histological staining by Hematoxilin-Eosin (HE).

Immunofluorescence: Indirect immunofluorescence (IFI) was used to observe the infected cells. Positive and negative sera for anti-HIV-1 antibodies were tested with the set of reagents of the IFI-HIV-1 kit produced at Biomanguinhos. IFI was performed by standard procedures: 1) Coverslips placed on the culture plates contained HIV-1-infected cells; 120 hours after virus inoculation, the coverslips were washed with buffered saline solution and fixed in acetone at -20 ºC for 10 minutes. 2) After drying, the coverslips were analyzed for reactivity of sera positive and negative for anti-HIV antibodies by immunologic reaction for 30 minutes in a moist chamber. 3) After incubation, a series of three washes of five minutes each in buffered physiological saline was performed. 4) The next step consisted of the addition of fluorescein-labeled human serum (conjugate) over the first reaction performed on the coverslip, also for a period of 30 minutes followed by three washes as described in item 3. Finally, the coverslips were dried and mounted on a larger coverslip for reading under the immunofluorescence microscope (IM-35, Zeiss).

Viral load: The last three passages (9th, 10th and 11th) were tested for the presence of virus by assessing the increase in viral load using Nasba-Organon equipment. Two-mL samples of each of the passages mentioned above were subjected to analysis, along with other routine blood samples tested at Retrovirus Laboratory of the Instituto Adolfo Lutz. Viral load values were below the detection threshold for which the equipment was configured, but it was possible to observe an increase between the 10th and 11th passages from 20 to 79 copies**.**

Isolation of HIV-1*:* HIV-1-infected McCoy cells cultured for approximately 10 days were centrifuged at 400 x g/min. The supernatant was removed, distributed into Sorwall type centrifuge tubes and centrifuged at 17,000 rpm for three hours at 4 ºC . The pellet was resuspended in a total volume of 1 ml Tris-NaCl-EDTA (TNE) buffer and stored overnight at 4 ºC.

The virus was purified on a chromatography column packed with 10 ml Sepharose 4B at room temperature. The eluate came immediately after void volume, approximately between 2.0 to 2.5 ml. The eluate was then centrifuged again in a microcentrifuge at 14,000 rpm for 1:30 hours. After removal of the supernatant, the pellet was resuspended in 150 µl buffer + 0.5% Triton X-100 in CMF-PBS for separation of HIV-1 viral protein by SDS-PAGE[22].

Western-blotting*:* After viral protein separation by SDS-PAGE, the separated proteins were transferred to a nitrocellulose strip and submitted to enzymatic reaction with serum samples from HIV-positive and HIV-negative (control) patients previously tested with a diagnostic kit.

Evaluation of CD4 type receptors in McCoy cells by flow cytometry: Qualitative and quantitative measurements of human CD4 surface expression on McCoy cells were determined by flow cytometry using acquisition and analysis. Surface staining was performed on 50 µL of 10^5 cells/mL with satured amounts of mouse anti-human CD4 fluorescein isothiocyanate (FITC) (DAKOPATTS, code F 766) and mouse anti-human CD3/CD4/CD8 conjugated monoclonal antibodies (TRITEST, Becton Dickinson Immunocytometry Systems - BDICS). The TRITEST reagent, routinely used for CD4 phenotype was used as a second, distinct antibody from a different source for positive staining control.

Fluorescence was analyzed using a FACSort flow cytometer (BDICS) equipped with an argon ion 488 nm laser source. FICT emission was collected with a filter at 530 nm wavelength (FL1). Cell size and granulosity were evaluated morphometrically by analysis of dispersal of transmitted light and of dispersal of light at 90º, respectively collected with FSC and SSC photomultipliers. The collecting filters were calibrated electronically using Calibrite beads (BDICS) and the resources of the system. Acquisition parameters were standardized by manual fine adjustments.

The morphometric, immunophenotypic and isotypic characteristics of the labeled cells were studied by analysis of the acquired data with the CELL-QUEST software (BDICS).

RESULTS

Figure 1A shows infected McCoy cells with altered morphology compared to control cells (Fig. 1B). The immunofluorescence illustrated in Figure 1C demonstrates that the morphology of infected cell + positive serum present a more fluid morphology of the cytoplasm and the contours of the cell membrane (greenish color). The control cells (not infected) + positive serum show an intact membrane of reddish color (Fig. 1D).

Figure 2 shows the viral proteins present in the infected cells that were visualized on the nitrocellulose strip by Western blotting. The serologic reaction of band precipitation was performed with positive patient serum (Fig. 2B), and the protein produced on a larger scale was p24, while the glycoproteins gp160, gp120 and gp41 as were produced on a smaller scale. The p31, p55 and 51, p18 and p15 proteins were

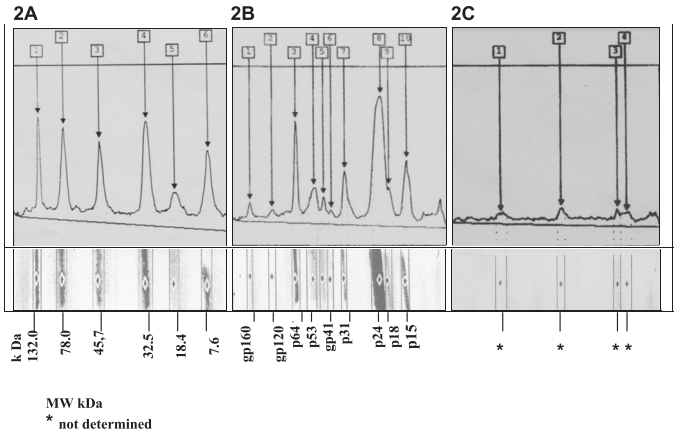

Fig. 2 - Shows the results of Western-blotting: the proteins obtained from infected McCoy cells after electrophoretic separation (SDS-PAGE) and transfer to cellulose strips were submitted to immunoprecipitation with serum from seropositive and seronegative patients with respective densitometries obtained with the Labscan equipment (Pharmacia-Biotech). (A) Prestained molecular weight standards from Biorad, # 85611- MW 7 -132 kDA-1)132 kDa; 2)78 kDa; 3)45.7 kDa; 4)32.5 kDa; 5)18.4 kDa and 6)7.6 kDa.; (B) serologic reaction of the nitrocellulose strip with the infected McCoy cell + positive serum 1) gp160; 2) gp120; 3) p64; 4) p55; 5) p53; 6) gp41; 7) p31; 8) p24; 9) p18; 10) p15 and (C) the same reaction but with a negative serum.

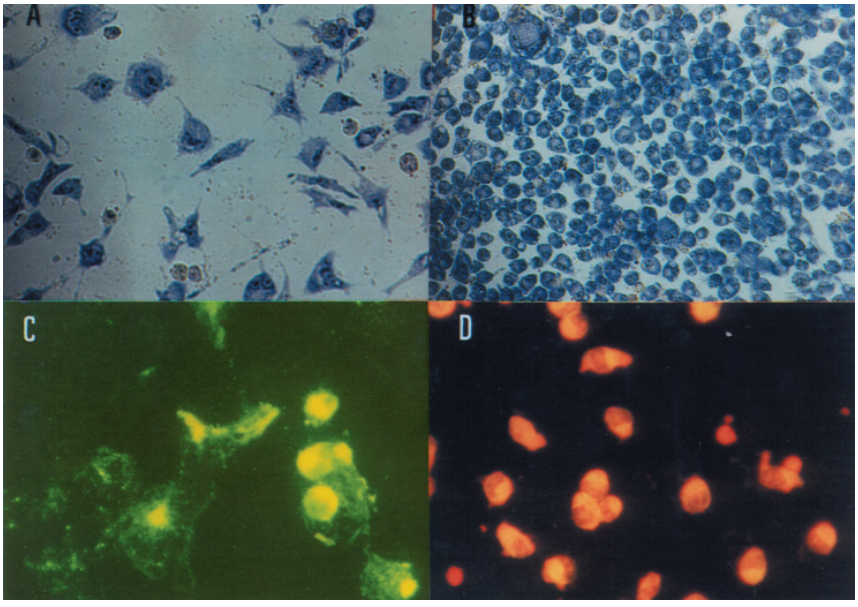

Fig. 1 - Shows photomicrographs of infected (A) and control (B) cells stained with hematoxylin-eosin obtained 120 hours after HIV-1 inoculation. Both cells showed a predominance of eosinophilia (bluish color). C and D cells are also infected and control cells visualized by immunofluorescence. In these photographs, the observation was made at higher magnification and the infected cells (C) show a greenish staining typical of positive reactions, whereas the control cells (D) show reddish staining.

present, and all proteins present were encoded by the major genes corresponding to the pol-env-gag loci of HIV-1[7].

In the reaction with negative serum, no proteins with the same molecular weight as the proteins cited above were identified (Fig. 2C).

The result of densitometry of the bands precipitated on the nitrocellulose strip, which characterizes the molecular weights of each band and the quantity of their virus replication production.

Figure 3 shows the result of the hypothesis raised. McCoy cells have CD4 receptors as proven by flow cytometry, which was used to measure the quantity of cells bound to the fluorescein-labeled CD4 antibody. These results were confirmed by the statistical significance of the non-parametric Kolgomorov-Smirnov test[45] (Table 1).

Table 2 shows the quantity of cells bound to the fluorescein-labeled CD4 antibody *versus* the inespecific Fc fraction isotypic mouse IgG1 bound to the cells, the results were also confirmed by the statistical significance of the non-parametric Kolgomorov-Smirnov test[45].

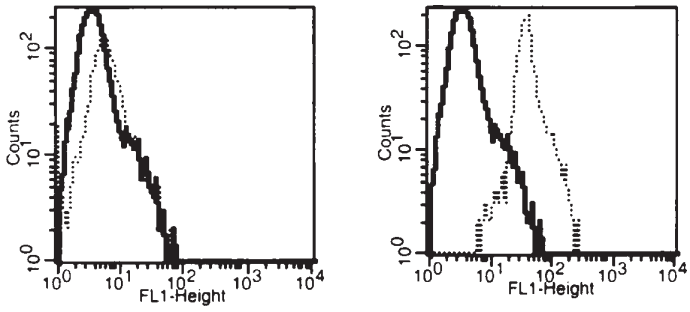

Fig. 3 - Shows the results of flow cytometry carried out to determine the presence of CD4 receptors in cells of the McCoy lineage. Morphometric analysis of McCoy cells using a bidirectional histogram (dot plot) of FSC and SSC revealed a homogeneous population of relatively small size and of moderate granulosity (Figures not shown). The expression of fluorescence in FL1 was evaluated. The presence of CD4 expression was evaluated in Figure 3: (A) control cells (not infected) + BSS without antibody *versus* uninfected cells, labeling with anti-CD3/CD4/CD8(¾/- -); (B) shows control cells + BBS *versus* uninfected cells labeling with the monoclonal antibody anti-CD4(¾ /- -).

DISCUSSION

The change in morphology was shown by an increase in area and in granulosity both in the cytoplasm and inside the nucleus, which was not observed in non-infected control cells[1,6]. According to these authors and others[31], the increase in permeability may be due to the concentration of intracellular Ca++.

This result shows why McCoy cells can isolate HIV-1 from plasma of seropositive patients and can also replicate the virus. This was verified by analyzing samples of passages of infected cell fluid in terms of viral load. In the 11th passage it was possible to measure 79 copies in 2 ml, which is equivalent to approximately 40 copies/ml, a much higher value than that reported later[5,35], the authors estimated less than 20 copies/ml to be the capacity of a cell of the organism of HIV-1-infected patients during the phase of clinical manifestation of the disease. Thus, data observed in the present study show the ability of McCoy cells to produce at least twice the amount of virus (viral load methodology), which makes McCoy cells a possible model for *in vitro* study of the dynamics of HIV-1 infection.

This cell also presents human CD4 receptors, which probably

Table 1
Cells parameters, # events, mean, median and standard deviation

Parameters	Events	Mean (C.I.)	Median (C.I.)	sd
1) Control Cell without Ab	19033	3.65 (3.64 - 3.68)	3.31 (3.27 - 3.35)	1.86
2) Cells * + Ab CD3/4/8	13247	6.06 (6.02 - 6.08)	5.68 (5.67 - 5.69)	2.38
3) Cells * + MAb CD4	8944	39.16 (39.08 - 39.240)	36.85 (36.77 - 36.93)	11.64

(C.I.) Confidential Interval; * Cells not infected.
The fluorescence intensities in FL1 were compared using as parameters the mean of the values attributed to light intensity and tested in each channel, the anti-CD4 antibody was found to label more receptors (mean = 39.16) than the anti-CD3/CD4/CD8 antibody (mean = 6.06). The light emission captured by the two cell populations presented a distribution close to normal, although with different mean and median values. The non- parametric Kolgomorov-Smirnov test was used for statistical analysis and the differences between control cells + BBS and cells + anti-CD4 were significant at $p < 0.001$, $\alpha = 0.05\%$.

Table 2
Cells parameters, # events, mean, median and standard deviation

Parameters	Events	Mean (C.I.)	Median (C.I.)	sd
1) Cells* + CD4 Ab	2770	21.95 (21.73 – 22.17)	20.54 (20.32 – 20.76)	5.92
2) Cells* + isotypic Ab	6367	7.79 (7.69 – 7.79)	7.77 (7.67 – 7.87)	3.89

(C.I.) Confidential Interval; * Cells not infected.
The fluorescence intensities in FL1 were compared using as parameters the mean of the values attributed to light intensity and tested in each channel, the anti-CD4 antibody was found to label more receptors (mean = 23.95) than the anti-mouse IgG1 (isotypic antibody) (mean = 3.69). The light emission captured by the two cell populations presented a distribution close to normal, although with different mean and median values. The non- parametric Kolgomorov-Smirnov test was used for statistical analysis and the differences between McCoy cells + isotypic antibody and McCoy cells + anti-CD4 were significant at $p < 0.001$, $\alpha = 0.05\%$.

increases its capacity to replicate HIV-1. The McCoy cells used in this study are hybrid cells containing chromosome markers both for human cells and mouse cells (ATCC)[2,12,36].

However, in order to confirm that results obtained with anti-CD4 marking were specific for the receptor and not an unspecific reaction of the Fc fraction of the IgG molecule, an experiment was also performed using isotypical mouse antibodies of the same type as monoclonal human anti-CD4 antibodies. The results shown in Table 2 reveal that there is marking for isotypical antibodies, but there is much more marking for anti-CD4-receptor antibodies.

However, this cell may well express part of its murine genome, since murine cells present a reduced capacity to replicate HIV-1. Cells of mouse lineages such as Rat2 and Nb2 have shown a capacity to produce the virus from 10 to 60 times less than human cells[20].

Some studies[20,28,40] have shown murine cell capacity for HIV-1 infection, in other words, the capacity for the virus to penetrate the cell, the integration and expression of products in the early and late stages, and the viral egress at different levels depending on the type of cell and its animal origin.

The advent of transgenic cells has enabled expression of human CD4, CCR5 co-receptors and T1 cycline molecules in murine cells, which have successfully replicated HIV-1 *in vitro*[28,37]. However, transgenic cells have shown a certain incapacity to liberate HIV[37]. This may explain why it has only been possible to observe HIV-1 proteins replicated in McCoy cells after treatment with lysis buffer containing EDTA to promote cellular lysis; the best results were obtained when this solution was immersed in an ultrasound bath. These results were observed in tests with elution of infected cells Fast Protein Liquid Chromatograph (FPLC) equipment to verify the molecular weight of the proteins extracted from the cells. This treatment has enabled observation of peaks of expression from gp160 to gp120 greater than the peaks observed when the cells were not subjected to this (data not demonstrated).

It remains to be determined whether the low glycoprotein (gp160 and gp120) concentration is due to the extraction method or if it is related to the concentration of CD4 receptors existing in McCoy cells that might sequester the glycoproteins (gp160) formed *de novo*, being reintegrated with the cell membrane and forming a conformational reaction through the gp120/41 complex (non-covalent association) expressed on the cell membrane[9,48]. This complex may join other CD4 receptors of nearby cells, forming syncytia[25,26,46]. This is a high-affinity interaction, so that the method used in the process may impair extraction since it would not be efficient in loosening the glycoproteins and another part would be lost in the cell debris. A definitive statement cannot be made at present, but this analysis will be the subject of a future study.

In the present study no comment on the actions of the CCR5 and CXCR4 co-receptors (chemokines)[3] or on the action of fusins in the mechanism of syncytium formation[51,53,54] was presented since no specific experiment to evaluate the presence of these receptors in McCoy cells was performed.

In conclusion, the present results permitted visualization of some important biological phenomena such as HIV-1 replication, the formation of giant cells, membrane permeabilization, and the presence of receptor CD4, which occur in McCoy cells infected with HIV-1. These observations could represent a good model to study the dynamics of viral replication and drug action or to induce virus modification (recombinant virus, genetic therapy) and observe the action mechanisms and cell function alterations *in vitro,* possibly in addition to vaccine therapy immune response trials and analyses.

RESUMO

Células de linhagem McCoy como um possível modelo contendo receptores CD4+ para estudos da replicação do HIV

Recentes estudos demonstraram o uso do vírus raiva como modelo vetor para produzir vacinas expressando as glicoproteínas do vírus HIV-1. A homologia na seqüência entre gp120 do vírus HIV-1 e a glicoproteína G do vírus rábico já foi previamente relatada. Devido a estes fatos a linhagem de célula McCoy utilizada com sucesso para a replicação do vírus rábico foi utilizada para demonstrar a replicação do HIV-1.

Amostra de HIV-1 foi isolada de plasma de um paciente soro positivo e inoculada em células de linhagem McCoy e então a infecção viral foi estudada em passagens sucessivas do vírus nesta célula. As proteínas liberadas no meio extra celular foram analisadas quanto a atividade biológica pela técnica de eletroforese em gel de poliacrilamida e imunotransferência em membrana de nitro-celulose reagindo com soros positivos para HIV-1 e soros de pacientes negativos.

As passagens sucessivas do HIV-1 em células demonstraram a replicação viral, o aumento da permeabilidade da membrana citoplasmática, a formação de sinsício e lise celular.

Análises com citometria de fluxo mostraram com clara evidência a presença de receptores CD4+ o que possivelmente deve ser a causa que possibilita a facilidade do isolamento e replicação do vírus HIV-1 nesta célula.

Concluindo os resultados observados permitem utilizar esta linhagem celular como um possível modelo para isolamentos de HIV, bem como realizar estudos da dinâmica de infecção viral em diversas situações inclusive de exposição a drogas em estudos farmacológicos, e talvez estudos e análises da resposta imune em terapias vacinais.

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