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PRODUCTION OF TNF-α BY PRIMARY CULTURES OF HUMAN KERATINOCYTES CHALLENGED WITH *Loxosceles gaucho* VENOM

Ceila M. S. MÁLAQUE(1), Maria ORI(2), Sânia A. SANTOS(1) & Dahir R. ANDRADE(1)

SUMMARY

Primary cultures of human keratinocytes were challenged with increasing doses from 10 ng/mL to 2 µg/mL of *Loxosceles gaucho* venom, responsible for dermonecrotic lesion in humans. TNF-α was investigated by bioassay and ELISA in the supernatant of the cultures challenged with 100 ng/mL, 500 ng/mL, 1 and 2 µg/mL of venom. TNF-α was detected by bioassay in the supernatant of cultures challenged with 100 ng/mL, after 6 h. The cytokine was detected by ELISA in the supernatant of the cells challenged with doses of l µg/mL, after 6 and 12 h. The results point out the capacity of this venom to activate the keratinocytes in primary cultures to produce TNF-α. The production of cytokines could contribute to the local inflammatory process in patients bitten by *Loxosceles sp*.

KEYWORDS: *Loxosceles*; Keratinocytes; Cell culture; TNF-α

INTRODUCTION

Loxosceles spider causes both a cutaneous necrosis and, less frequently, a cutaneous-visceral form of envenomation in humans where, besides the local lesion, hemolysis is also present. The bite is relatively painless and only 2-8 h after bite patients complain of pain. There may be transient erythema, swelling and mild to severe tenderness. The erithema turns to a violaceous color. An scar may form, between 3 and 7 days, and drop off, leaving an ulcer that heals in varying lengths of time (6-8 weeks), but sometimes also require skin grafting[9].

Histopathologic changes include edema and thickening of endothelium of blood vessels, collections of inflammatory cells, vasodilation, intravascular coagulation, hemorrhage into the dermis and even into the subcutis. The accumulation of polymorphonuclear leucocytes (PMNs) is especially marked[27,28].

Several purified components of *Loxosceles* venom have been identified. The most important component is sphingomyelinase D, a 32-35 kDa fraction that may produce necrotic lesions, hemolysis of red blood cells and death of experimental laboratory animals[2,3,4,18,23]. The kind of sphingomyelinase (SMase) of *Loxosceles* venom that possesses an optimal activity at pH 7.1[8], allows us to characterize it as a neutral sphingomyelinase. This sphingomyelinase hydrolyses the sphingomyelin of the cellular membrane, originating the sphingolipid ceramide[12,13].

PATEL *et al.*[26] demonstrated that *L recluse* venom activates endothelial cells that express selectin E (CD62a) and causes liberation of IL-8 and GM-CSF. MODUR *et al.*[22], studying endothelial cells in primary culture, observed that both the synthetic ceramide (C8-ceramide) and that produced by the action of exogenous sphingomyelinase (sphingomyelinase C) activate synthesis of adhesion molecules by endothelial human cells, delimiting the inflammatory process. Ceramide, in a similar way to the sphingomyelinase D of *Loxosceles* venom, induces the endothelial cells to link to the activated neutrophils, through the selectin E and of IL-8. This phenomenon helps explain the exuberant presence of neutrophils in the dermonecrotic lesions of *Loxosceles* envenomation.

An important aspect of the phenomenon originates in the epidermis because it is where the venom takes place primarily, in the transition of the layer epiderm-derm. In this context, it is justifiable to choose the keratinocyte as one of the target cells, based on evidences of NICKOLOFF & TURKA[25], that have established the mechanisms of exogenous aggression of the skin by physical agents as ultraviolet, contact allergens, microorganisms, etc. Activated keratinocytes convert exogenous stimuli, by appropriate transcription factors, in coding cytokines genes, adhesion molecules and chemotatic factors responsible for the initiation of the cutaneous inflammation[1,5,17,21,25].

Abbreviations: ICAM-1: Intercellular adhesion molecule; IL-1: Interleukin-1; IL-8:Interleukin-8; GM-CSF: Granulocyte-macrophage colony-stimulating factor; LPS: Lipopolysaccharide; MHC II: Major histocompatibility complex II; UV: Ultraviolet; VCAM-1: vascular cell adhesion molecule 1.This work was supported by CNPq and CAPES.

(1) Laboratório de Investigação Médica (LIM 54), Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (HCFMUSP), São Paulo, SP, Brasil.
(2) LIM 12, HCFMUSP, São Paulo, SP, Brasil.
Correspondence to: Ceila Maria Sant'Ana Málaque, Hospital Vital Brazil, Instituto Butantan. Av. Vital Brazil 1500, 05503-900 São Paulo, SP, Brazil. Phone: (011) 3061-9809. e-mail: naputano@uol.com.br

The objective of this study was to describe the keratinocytes cultures and to assess the presence of TNF-α in the supernatant of human keratinocytes cultures challenged with *Loxosceles gaucho* venom.

MATERIAL AND METHODS

Venom: Venom collected from the fangs of *Loxosceles gaucho* by electrical stimulation of the spider was provided by Section of Venom of Instituto Butantan, obtained by electrostimulation by the method of BÜRCHEL[7], with slight modifications, lyophilized and stored at -20 °C. It was diluted in saline, aliquoted and stored at -20 °C.

Cell Culture: Foreskins obtained from routine circumcisions were washed in 70% alcohol for 20-30 s and transferred to a medium containing high concentration of antibiotics, for 15 min[31]. After separating derma/epidermis mechanically, the tissue was cut into pieces of 1 x 1 mm and placed into 25 cm^2 plastic flasks (Nunc). After 22 h at 37 °C and in 5% CO^2, 4 mL of the culture medium (K-SFM; Gibco) was carefully added to ensure that all pieces were covered by culture medium. The flasks were then incubated at 37 °C and with 5% CO_2. After 5 days, the flasks were inspected to observe epidermal cell growth. The culture medium was replaced 2 to 3 times per week up to the confluence of the cells.

Loxosceles gaucho venom diluted in RPMI-1640 (Sigma) and concentrations of 10 ng/mL, 100 ng/mL, 200 ng/mL, 500 ng/mL, 1 µg/mL e 2 µg/mL were added to monolayer cultures of keratinocytes which were inspected 6, 12, 24 and 36 h after challenge to assess the venom effects.

Electron microscopy: Cultures of keratinocytes exposed to 2 µg/mL of *L. gaucho* venom were detached by trypsin solution (trypsin 0.2%; EDTA in PBS 0.02% - Instituto Adolfo Lutz). The supernatant was centrifuged and glutaraldehyde was added to the pellet for 2 h. It was post-fixed in osmium 1%, block stained in 0.5% uranyl acetato, overnight, and dehydrated in a graded series of ethanol, acetone and araldite resin. The preparation was examined by electron microscopy (JEOL).

Microbiologic analysis of the contaminated cultures: Of those culture media that became turbid, 100 µL were removed, placed in tube contends TSB (Tryptic Soy Broth, DIFCO), and maintained by 24 h for 37 °C. Later, a plate of agar-blood was sowed (middle of Müeller-Hinton, DIFCO, with 5% defibrinated ovine red blood cells, Biotério Boa Vista) with darkened TSB. Identification of the microorganism was carried out by analysis of the morphologic characteristics of the colonies and Gram staining.

Bioassay for TNF-α: Confluent keratinocytes cells were treated with venom concentrations of 100 ng/mL, 500 ng/mL, 1 µg/mL and 2 mg/mL in RPMI-1640. After different incubation periods (0, 6, 12, 24, 36 h), an aliquot of cell culture supernatant was removed and TNF-α was investigated by bioassay. Briefly, L929 cells were seeded at the concentration of 3 x 10^4 cells/well of a 96-well, in 100 µL culture medium RPMI-1640 (Sigma), and incubated at 37 °C and at 5% CO_2 atmosphere to establish a dense monolayer. Actinomycin D was added at the concentration of 1 µg/mL and incubated for 2 h. Samples were tested in duplicate or triplicate. After 24 h incubation at 37 °C, in 5% CO_2, the plates were rinsed and stained with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), 500 µg/mL. The optical density (O. D.) was measured, after 1 h, at 595 nm on BIO-RAD microplate reader. Percentage viability was calculated using the formula:

$$\frac{\text{O.D. (test)}}{\text{O.D. (control)}} \times 100,$$

where control cells were incubated with culture medium only. Results were compared with a standard curve of human recombinant TNF-α and expressed as pg/mL.

ELISA: Confluent keratinocytes were exposed to venom at 500 ng/mL, 1 µg/mL and 2 µg/mL in RPMI-1640. After different incubation periods (0, 6, 12, 24, 36 h), an aliquot of cell culture medium was removed. Human TNF-α (hTNF-α) was investigated in the supernatant by a commercially available ELISA kit (Boehringer Mannheim). Detection of standard TNF-α was linear over the range 20-800 pg/mL. The limit of detection was ≥ 12 pg/mL and limit of quantification was ≥ 20 pg/mL.

RESULTS

Cell culture

Initially contamination was frequent and could be observed from the 4th day of culture, when the culture medium become turbid. The isolated microorganisms were *Candida spp*. and *Streptococcus spp*. We observed that the tissues of children ≥ 2 and < 3 year old children had a higher contamination rate (56.2%) when compared to other groups.

The beginning of the cellular growth occurred from the 7th to the 10th day of culture. Cells had a polygonal format, with large nucleus, prominent nucleoli and cytoplasm with variable number of granules. By electron microscopy, organelle and cytoplasmic filaments were observed in the perinucleus, and intercellular contacts by microvilli.

Development of monolayer, necessary to allow the addition of the venom, occurred about the 21st day. With K-SFM (GIBCO), no contamination was observed with fibroblasts in the primary cultures. However, after two passages of some cultures, there was an exuberant growth of fibroblasts not allowing the continuation of the subcultures.

Effects of *Loxosceles gaucho* venom on the keratinocytes cultures

There were no changes in cell morphology assessed by optical microscopy after 36 hour-contact with the venom at concentrations of 10 to 500 ng/mL. Even challenging the cells with 10 ng/mL to 200 ng/mL of venom, cells continued to be cultured for 4 weeks, denoting its viability. On the other side, cells incubated with venom at 500 ng/mL died after 5 days. When using 1 and 2 µg/mL, after 36 h of contact with venom, the cells showed increase of granulation and cytoplasm vacuolation, cellular death and detached cells when compared to the control. Cells challenged with 2 µg/mL, observed by electron microscopy after 36 h of incubation, showed vacuoles increase in the cytoplasm.

Detection of TNF-α by bioassay and ELISA

By bioassay, 7.0 pg/mL of TNF-α was detected in the supernatants of keratinocyte cultures challenged with 100 ng/mL of the toxin for 6 h. With the other concentrations, we did not detect the presence of TNF-α.

By ELISA, TNF-α was detected, 6 and 12 h after challenge with 1 µg/mL of venom (Fig. 1). With 2 µg/mL, the levels were below of the limit of quantification of the test (T36 = 18 pg/mL) (Fig. 2); with 500 ng/mL, no TNF-α was detected.

DISCUSSION

The choice of human keratinocytes as target cells to the action of *L. gaucho* toxin is based on two different aspects. One is the hypothesis that the deposition of the venom occurs in the transition between epidermis and derma. The other is the fact that keratinocytes have, beside their coating function, important immunological functions, reacting to external insult with cytokine production (TNF-to, IL-1, IL-8), expression of adhesion molecules (ICAM-1, VCAM-1) and of molecules of the MHC II[5,24,25]. Cellular culture could facilitate the study of the interaction of toxin and cells, thus allowing to evaluate its behavior when challenged by toxins.

Some considerations could be made about the primary cultures of keratinocytes. Contamination was a limiting factor to the development of the culture. The source of contamination was the tissue obtained from foreskin of children submitted to circumcision. The isolated microorganisms were *Streptococcus sp.* and *Candida sp*, present in the flora of genital area. The infections were more frequent in the tissues of 2 to 3 year old-children, the age group where that surgery is mostly indicated.

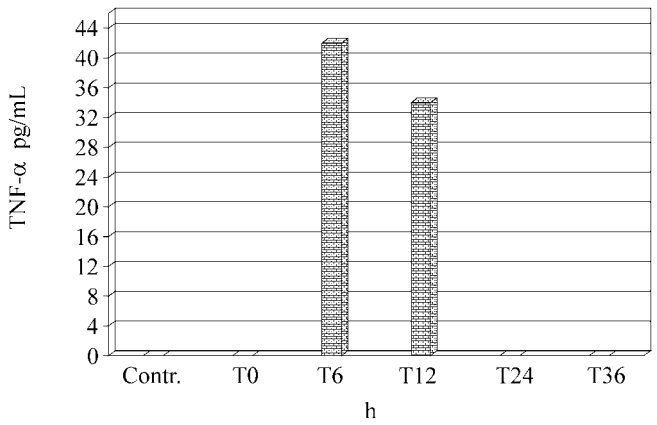

Fig. 1 - TNF-α by ELISA in supernatant of keratinocytes challenged with L. *gaucho* venom - 1 µg/mL.

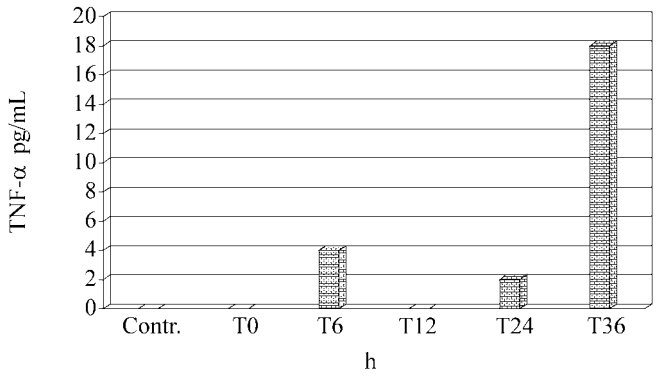

Fig. 2 - TNF-α by ELISA in supernatant of keratinocytes challenged with L. *gaucho* venom - 2 µg/mL.

The cellular growth began from the 7th to the 10th day. In 2 to 3 weeks, the cells were confluent, forming a monolayer as a pavement, with large nucleus and prominent nucleoli, as referred in the literature[29,31,32]. The visualization of the cells by electron microscope revealed cells with perinuclear filaments, similar to those previously described[29]. As observed in cultures of mice keratinocytes[14], the intercellular contacts are made by microvilli, probably because the culture medium contained low concentrations of calcium (0.09 mM Ca++), that inhibited the desmosomes formation.

When the cultures formed a cellular monolayer reaching 60-70% of the bottle, the cells were challenged with the toxin. Increasing doses of venom were used, from 10 ng/mL until 2 µg/mL with the purpose of observing the behavior of the cells. With doses of 500 ng/mL to 2 µg/mL, cellular damage and death were observed, occurring earlier with larger doses. The difficulty to obtain a subculture, as referred previously[6], did not allow us to make tests of cellular viability, time and dose dependent to the challenge with the venom.

The levels of TNF-α detected in the supernatant by ELISA were comparable to those described when the keratinocytes were stimulated with UV[16] and 1α,25dihydroxyvitamin D3[11]. With 1µg/mL of venom, 42 pg/mL and 34 pg/mL of TNF-α were detected 6 and 12 h after challenge, respectively. It is interesting to notice that either by bioassay or ELISA, TNF-α was detected 6 hours after challenge and it is possible that the detection could be made earlier as referred by GEILEN *et al.*[11].

The presence of the TNF-α, observed in some experiments accomplished with keratinocytes cultures, may contribute to the understanding the role of cytokines to localize the inflammatory process, as mentioned by NICKOLOFF & TURKA[25]. The eventual presence of ceramide in the endothelial cells, resulted from *Loxosceles* sphingomyelinase activity[26], associated to TNF-α production by keratinocytes, could explain the synthesis of adhesion molecules and the intense inflammatory deviation that are observed in the lesions.

The *L. gaucho* venom activity on the keratinocytes reveals its potentiality as agent of cellular death. GEILEN *et al.*[11], using permeable ceramide in cellular lineage of human keratinocytes (HaCaT), observed apoptosis induction, measured by fragmentation of DNA and electron microscopy. The action of the ceramide, resultant of the sphingomyelin's cleavage of by sphingomyelinase, may lead to death by apoptosis (a pathway involving caspases) or necrosis. The TNF-α, a powerful cytotoxic agent, is also responsible for cellular death, using as intracellular second messenger the ceramide, that leads cytocrome **c** production and activation of caspases[15,19,20,30]. Therefore, it is possible that, in the lesion caused by *Loxosceles* spider, signs triggered by ceramide and action of TNF-α result in cellular death by necrosis and/or apoptosis[10,19,21].

RESUMO

Produção de TNF-α por queratinócitos humanos em cultura primária após agressão com veneno de *Loxosceles gaucho*

Culturas primárias de queratinócitos humanos foram incubadas com veneno de aranha *Loxosceles gaucho*, que possui atividade esfingomielinase D, responsável por lesão dermo-necrótica nos acidentes humanos. As células das culturas primárias foram agredidas com o veneno em doses crescentes de 10 ng/mL a 2 µg/mL. No sobrenadante das culturas agredidas com 100 ng/mL, 500 ng/mL, 1 e 2 µg/mL da toxina, foi pesquisada a presença de TNF-α através de bioensaio e ELISA. Com 100 ng/mL, foi detectado TNF-α no sobrenadante após 6 h, no bioensaio; usando o teste de ELISA, detectou-se a citocina no sobrenadante de células agredidas com doses de 1 µg/mL, após 6 e 12 h. Os resultados apontam para a capacidade deste veneno em ativar os queratinócitos em cultura, levando-os a produzir TNF-α. É provável que a produção de citocinas ative as células endoteliais, auxiliando na localização do processo inflamatório.

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REFERENCES

1. ANSEL, J.; PERRY, P.; BROWN, J. *et al.* - Cytokine modulation of keratinocyte cytokines. **J. invest. Derm**., **94:** 101S-107S, 1990.

2. BARBARO, K.C**. - Caracterização biológica, imunoquímica e bioquímica do veneno de aranhas do gênero** *Loxosceles*. São Paulo, 1996. (Tese de Doutoramento - Instituto de Ciências Biomédicas da Universidade de São Paulo).

3. BARBARO, K.C.; CARDOSO, J.L.C.; EICKSTEDT, V.R.D. & MOTA, I. - Dermonecrotic and lethal components of *Loxosceles gaucho* spider venom. **Toxicon**, **30:** 331-338, 1992.

4. BARBARO, K.C.; FERREIRA, M.L.; CARDOSO, D.F.; EICKSTEDT, V.R.D. & MOTA, I. - Identification and neutralization of biological activities in the venoms of *Loxosceles* spiders. **Braz. J. med. biol. Res., 29:** 1491-1497, 1996.

5. BARKER, J.N.W.N.; MITRA, R.S.; GRIFFITHS, C.E.M.; DIXIT, V.M. & NICKOLOFF, B.J. - Keratinocytes as initiators of inflammation. **Lancet**, **337:** 211-214, 1991.

6. BREIDAHL, A.F.; JUDSON, R.T. & CLUNIE, G.J.A. - Review of keratinocytes culture techniques: problems of growing skin. **Aust. N. Z. J. Surg**., **59:** 485-497, 1989.

7. BÜRCHEL, W. - Biology and venoms of the most important South American spiders of the genera Phoneutria, Loxosceles, Lycosa and Latrodectus. **Amer. Zool**., **9:** 157-159, 1969.

8. FORRESTER, L.J.; BARRET, J.T. & CAMPBELL, B.J. - Red blood cell lysis induced by the venom of the brown recluse spider. The role of sphingomyelinase D. **Arch. Biochem., 187:** 355-365, 1978.

9. FUTRELL, J. M. - Loxoscelism. **Amer. J. med. Sci**., **304:** 261-267, 1992.

10. GAMARD, C.J.; DBAIBO, G.S.; LIU, B.; OBEID, L.M. & HANNUN, Y.A. - Selective involvement of ceramide in cytokine-induced apoptosis. Ceramide inhibits phobol ester activation of nuclear factor kappaB. **J. biol. Chem., 272:** 16474-16481, 1997.

11. GEILEN, C.C.; BEKTAS, M.; WIEDER, T. *et al.* - 1α,25-Dihydroxyvitamin D_3 induces sphingomyelin hydrolysis in HaCaT cells via tumor necrosis factor α. **J. biol. Chem., 272:** 8997-9001, 1997.

12. HANNUN, Y.A. & BELL, R.M. - Functions of sphingolipids and breakdown products in cellular regulation. **Science, 243:** 500-507, 1989.

13. HANNUN, Y.A.; OBEID, L.M. & WOFF, R.A. - The novel second messenger ceramide: identification mechanism of action, and cellular activity. **Advanc. Lipid Res., 25:** 43-64, 1993.

14. HENNINGS, H.; MICHAEL, D.; CHENG, C. *et al.* - Calcium regulation of growth and differentiation of mouse epidermal cells in culture. **Cell, 19:** 245-254, 1980.

15. JARVIS, D.W.; GRANT, S. & KOLESNICK, R.N. - Ceramide and the induction of apoptosis. **Clin. Cancer Res., 2:** 1-6, 1996.

16. KÖCK, A.; SCHWARZ, T.; KIRNBAUER, R. *et al.* - Human keratinocytes are a source for tumor necrosis factor alpha: evidence for synthesis and release upon stimulation with endotoxin or ultraviolet light. **J. exp. Med., 172:** 1609-1614, 1990.

17. KUPPER, T.S. - The activated keratinocyte: a model for inducible cytokine production by non-bone marrow-derived cells in cutaneous inflammatory and immune responses. **J. invest. Derm., 94:** 146S-150S, 1990.

18. KURPIEWSKI, G.; FORRESTER, L.J.; BARRET, J.T. & CAMPBELL, B.J. - Platelet aggregation and sphingomyelinase D activity of a purified toxin from the venom of *Loxosceles reclusa*. **Biochim. biophys. Acta (Amst.), 678:** 467-476, 1981.

19. LEMASTER, J.J. – The mitochondrial permeability transition: from biochemical curiosity to pathophysiological mechanism. **Gastroenterology, 115:** 783-786, 1998.

20. MATHIAS, S. & KOLESNICK, R. - Ceramide: a novel second messenger. **Advanc. Lipid Res., 25:** 65-90, 1993.

21. McKENZIE, R.C. & SAUDER, D.N. - The role of keratinocyte cytokines in inflammation and immunity. **J. invest**. **Derm., 95:** 105S-107S, 1990.

22. MODUR, V.; ZIMMERMAN, G.A.; PRESCOTT, S.M. & McINTYRE, T. - Endothelial cell inflammatory response to tumor necrosis factor α. **J. biol. Chem**., **271:** 13094-13102, 1996.

23. MOTA, I. & BARBARO, K.C. - Biological and biochemical properties of venoms from medically important *Loxosceles* (Araneae) species in Brazil. **J. Toxicol.-Toxins Rev**., **14:** 401-421, 1995.

24. NICKOLOFF, B.J. & NAIDU, Y. - Perturbation of epidermal barrier function correlates with initiation of cytokine cascade in human skin. **J. Amer. Acad. Derm**., **30:** 535-546, 1994.

25. NICKOLOFF, B.J. & TURKA, L.A. - Keratinocytes: key immunocytes of the integument. **Amer. J. Path**., **143:** 325-331, 1993.

26. PATEL, K.D.; MODUR, V.; ZIMMERMAN, G.A.; PRESCOTT, S.M. & MCINTYRE, T.M. - The necrotic venom of brown recluse spider induce dysregulated endothelial cell-dependent neutrophil activation. **J. clin. Invest., 92:** 631-642, 1994.

27. PIZZI, T. - Estudios histopatológicos del aracnidismo necrótico por *Loxosceles laeta*. **Bol. chil. Parasit**., **30:** 34-36, 1975.

28. REYES, V. & ATIAS, A. - Un caso clinico de loxoscelismo cutaneo visceral. **Bol. Hosp. S. Juan (Santiago), 28:** 331-336, 1981.

29. ROSE, B.R.; THOMPSON, C.H.; MCDONALD, A.M. *et al.* - Cell biology of cultured anogenital warts. **Brit. J. Derm., 116:** 311-322, 1987.

30. SMYTH, M.; OBEID, L.M. & HANNUN, Y.A. – Ceramide: a novel lipid mediator of apoptosis. **Advanc. Pharmacol., 41:** 133-154, 1997.

31. THOMPSON, C.H.; ROSE, B.R. & COSSART, Y.E. - Optimised growth of human epidermal cells in vitro without the use of a feeder layer or collagen substrate. **Aust. J. exp. Biol. med. Sci., 63:** 147-156, 1985.

32. VIEIRA, K.B.L. - **Cultura de células epiteliais normais ou transfectadas por DNA viral, como um modelo de estudo de Papilomavírus humano**. São Paulo, 1991. (Dissertação de Mestrado - Departamento de Bioquímica e Biologia Celular da Escola Paulista de Medicina).

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