EXPERIMENTAL STUDY

HLA-G1-transfected K562 cells do not inhibit NK-cell-mediated lysis in europium release cytotoxicity assay

Sapak M, Buc M

*Department of Immunology, Faculty of Medicine, Comenius University, Bratislava, Slovakia.***michals@pobox.sk**

Abstract

The class Ia of HLA molecules are recognised by NK-cells either by inhibitory or stimulatory NK-receptors. When inhibitory signals prevail over stimulatoryones, the target cells expressing the class Ia of HLA molecules are not lysed by NK-cells. Similarly, class Ib of HLA molecules have been reported to induce the inhibitory signal in NK-cells. The cell line K562 is deprived of both class Ia and class Ib of HLA molecules, respectively, the fact of which enhances the lysis of K562 cells when they are co-cultivated with NK-cells. To study the role of HLA-G molecules in NK-cell cytotoxic activity, HLA-G tranfected K562 cells were used as target cells. NK-cells were isolated from the peripheral blood of 4 unrelated persons using Miltenyi's Biotec isolation system. The purity of directly isolated NK cells (CD56 Multisort kit) was 74.1 %, and that of indirectly isolated NK-cells (NK-cell isolation kit) 69.4 %. The europium release cytotoxicity assay was used in all experiments. The percentage of cytotoxicity ranged from 19 % to 24 % when K562 target cells were used. Similar results were obtained with the HLA-G1-transfected target cells: the percentage of cytotoxicity ranged from 17 % to 29 %. Our preliminary results indicate that NK-cells are able to lyse both, K562 cells and the HLA-G1-transfected K562 cells. ***(Tab. 1, Fig. 8, Ref. 21.)***

Key words: NK-cells, HLA-G molecules, NK-cell mediated cytotoxicity, Europium release assay.

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NK cells represent an independent population of lymphocytes. They recognise the class I of HLA molecules and other cell surface structures either by inhibitory or stimulatory NK-receptors. When inhibitory signals prevail over the stimulatory, the target cells expressing the class Ia of HLA molecules are not lysed by NK-cells. Similarly, the class Ib of HLA molecules have been reported to induce the inhibitory signal in NK-cells.

The HLA-G molecules belong to class Ib (non classical) molecules. They differ from classical HLA-Ia molecules by: I) alternative splicing of their mRNAs encoding for at least four membrane-bound and two soluble HLA-G isoforms, II) limited polymorphisms, and III) tissue-restricted distribution (1). The HLA-G1 molecules are preferentially expressed in syncytiotrophoblasts to protect the HLA-semialogeneic foetus from the decidual NK-cell-mediated lysis (2, 3, 4).

Cells of the human erythroleukemia cell line K562 are deprived of HLA molecules which makes them a suitable target to test their NK-cell mediated cytotoxic activity (5). Some previous works have shown that HLA-G1-transfected K562 cells resist to the NK-cells cytotoxicity (4, 6, 7, 8). Moreover, similar results have been observed with the HLA-G1, -G2, -G3 and -G4-transfected melanoma cell line M8 (7). However, some other reports contradict these results (9). The aim of our work was therefore to contribute to the resolution of the problem by investigating the NK-cell cytotoxicity using HLA-G1-transfected K562 target cells.

Methods

Effector cells: Peripheral venous blood was collected from 6 blood donors (aged 30–50 years) into heparinized tubes and lym-

Department of Immunology, Faculty of Medicine, Comenius University, Bratislava

Address for correspodence: M. Sapak, MD, Dept of Immunology LFUK, Sasinkova 4, SK-811 08 Bratislava 1, Slovakia.
Phone: +421.2.59357.204, Fax: +421.2.59357.578

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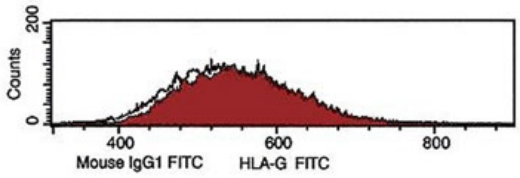

Fig. 1. The expression of HLA-G1 molecules in membrane of K562 cells detected by flow cytometry: Histograms of isotype-matched negative control (mouse IgG1-FITC) and surface membrane structure stained with anti-HLA-G1-FITC monoclonal antibody. Abscissa — intensity of immunofluorescence, ordinate — number of cells.

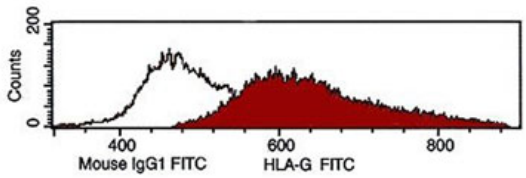

Fig. 2. The expression of HLA-G1 molecules in membrane of K562 transfected cells detected by flow cytometry: Histograms of isotype-matched negative control (mouse IgG1-FITC) and surface membrane structure stained with anti-HLA-G1-FITC monoclonal antibody. Abscissa – intensity of immunofluorescence, ordinate – number of cells.

phocytes were isolated by density gradient centrifugation using the Ficoll-Hypaque solution (1.077 g/mL; Pharmacia Biotech, Uppsala, Sweden). The cells were washed once with PBS (phosphate-buffered saline), which was followed by washing them with MACS-buffer (PBS with 0.5 % (wt/vol) bovine serum albumin and 2 mM EDTA). NK-cells were isolated from PBMC using the MidiMACS System (Miltenyi Biotec GmbH, Gladbach, Germany). Two different isolation procedures for isolation of NK-cells were used; the manufacturer's instructions (Miltenyis Biotec GmbH, Gladbach, Germany) were followed meticulously. The first method used $CD56^+$ monoclonal antibodies conjugated with magnetic beads (CD56 Multisort Kit); the purification of NK cells by the second method was done indirectly by the depletion of T-cells, B-cells, and myeloid cells (NK-Cell Isolation Kit). Enriched NK-cells were first washed in PBS; the 2nd washing was performed in a complete culture medium (CM) (RPMI 1640 (Gibco/BRL, UK) supplemented by 2 mM L-glutamine, 100 IU/mL penicillin, 100 ng/mL streptomycin and 9 % foetal calf serum (FCS; Bio-Whittaker, USA). Washed cells were resuspended at the concentration of $0.08.10^6$/mL in CM. The purity of isolated NK-cells was estimated by flow cytometry (Immunocytometry Systems; Becton-Dickinson, San Jose, USA).

Target cells: The K562 cell line (American Type Culture Collection) and K562-HLAG1-transfectants were maintained in CM cultured in a 37 °C, 5 % CO_2 humified incubator. The expression of HLA-G1 in cell membranes of K562 cells was verified by FACS-scan analysis (Immunocytometry Systems; Becton-Dickinson, San Jose, USA). Two different monoclonal antibodies (mAb) were used: monomorphic anti-HLA-G1 labelled by FITC, and an isotype-matched mouse FITC IgG1. The K562-transfectants and anti-HLA-G monoclonal antibodies were kindly offered to us by V. Rebbman (Institute of Immunology, Essen).

Tab. 1. The percentage and purity of $CD16^+56^+$ NK-cells using two different isolation techniques.

	NK-CellIsolation Kit	CD56-Multisort Kit
Percentage of isolated NK-cells from PBMC (n=5)	7.4%	8.3%
Purity of isolated NK-cells from isolated mononuclear cells (n=5)	**69.4%**	**74.1%**

PBMC – peripheral blood mononuclear cells

The cells were split three days prior to the testing to ensure that they had been in the log phase. On the day of testing, the K562 cells were washed in saline to reduce the content of extracellular Ca^{2+}. One cell pellet was resuspended in 1 mL of a labelling buffer (50 mM HEPES, 93 mM NaCl, 5 mM KCl, and 2 mM $MgCl_2$ in 1 litre of distilled water adjusted to pH 7.4.) supplemented with 20 µM Eu $(DH_3C00)^{3+}$, 100 µM DTPA, and 1 mg of dextrane sulfate. The cell suspension was incubated for 25 min at room temperature with occasional shaking. The labelling process was stopped by addition of $CaCl_2$ (30 µL of 100 mM $CaCl_2$ solution per mL). After 5 min of continued incubation, the cells were washed four times in Repair buffer (50 mM HEPES, 93 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$ and 10 mM glucose in 1 liter of distilled water adjusted to pH 7.4.) and twice in CM. They were eventually resuspended in CM at a concentration of $0.1.10^6$/mL.

Europium release cytotoxicity assay: Aliquots of effector cells (0.1 mL) and target cells (0.05 mL) were dispensed in each well of a 96 V-bottomed microtiter plate (Sarstedt 83.1838.500, Germany). Twofold dilution was made to obtain an appropriate effector to target (E:T) ratio 16:1, 8:1, 4:1, 2:1. The microplates were centrifuged briefly to bring effectors and targets into close contact. The incubation time was 100 minutes at 37 °C in a humified atmosphere of 5 % CO_2 in the air. All assays were done in duplicates. The plates were centrifuged after incubation again and supernatants harvested to measure the released Europium. 20 µl aliquots of the supernatant were transferred to wells of a flat-bottom 96-well microplate (Cellstar**,** E & K Scientific Products, Inc.) and 200 µL of an enhancement solution (PerkinElmer-Wallac, Inc.) were added to each well. Fluorescence was measured in a time-resolved fluorometer (Arcus 1232 Delfia, LKB-Wallac) following 7 minutes of slow shaking. The percentage of specific lysis was calculated as follows:

$$\text{percentage of specific lysis} = \frac{(\text{cpm experimental release} - \text{cpm spontaneous release})}{(\text{cpm maximum release} - \text{cpm spontaneous release})} \times 100.$$

The spontaneous release was determined by incubation of labelled target cells in CM, the maximum release was achieved by thorough mixing of the target cells with a pipette. The spontaneous release was less than 15 % of the maximum release in all

experiments done. The results are presented as the mean of duplicate samples. All experiments were performed 3 times in a period of two weeks

Results

Two different methods were used to isolate $CD16^+CD56^+$ NK-cells in 5 healthy volunteers. The percentage of NK-cells of the whole peripheral blood ranged from 7.4 % using the NK Cell Isolation Kit to 8.3 % with the CD56 Multisort Kit. The same techniques used to purify NK-cells from previously isolated mononuclear cells yielded 69.4 % with the 1st technique and 74.1 % with the 2nd one, respectively (Tab. 1). NKT-cells ($CD3^+CD56^+$) contaminated the NK-Cell Isolation Kit isolated NK-cells and erytrocytes and granulocytes those purified with the CD56 Multisort Kit.

The level of HLA-G1 cell-surface expression in K562 cells and in K562-transfected cells respectively was verified by flow cytometry analysis. None of the anti-HLA-G1 mAbs bound to K562 control cells (Fig. 1). However, a high level of HLA-G expression was detected in the cell surface of K562-HLA-G1 transfectants (Fig. 2). The results confirmed that K562 transfected cells had expressed the HLA-G molecules and were suitable targets for NK-cell-mediated cytotoxicity tests.

Six tests were performed, the NK-Cell Isolation Kit NK-cells were used twice and the CD56 Multisort Kit four times. The mean percentage of cytotoxicity against the K562 target cells at the effector to target ratio of 8:1 was 19 % to 24 %. Similar results were obtained when HLA-G1-transfected target cells were used: the percentage of cytotoxicity ranged at the same effector to target cell ratio from 17 % to 29 % (Figs 3, 4, 5, 6). The spontaneous release of europium was lower than 15 % in all NK-cell cytotoxicity experiments. A one day old blood sample was used in two experiments that resulted in a decrease in the percentage of cytotoxicity (2–7 %), however the inhibitory effect of HLA-G1 was not observed either (Figs 7 and 8).

As the number of performed experiments was too small, the statistical evaluation of the results was not possible. However,

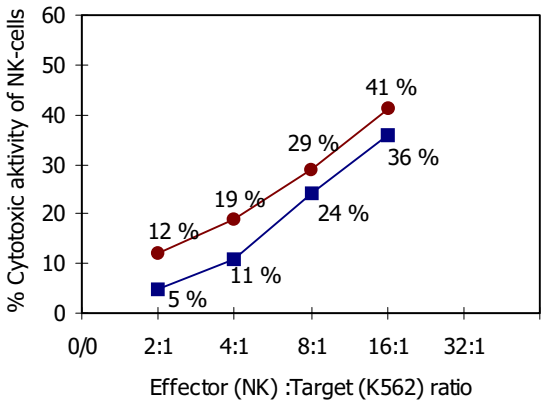

Fig. 3. The cytotoxic activity (y-axis) of the NK Cell Isolation Kit NK-cells against K562 (•) and HLA-G1 K562-transfected cells (o), respectively, in 4 different effector to target cells ratios (x-axis).

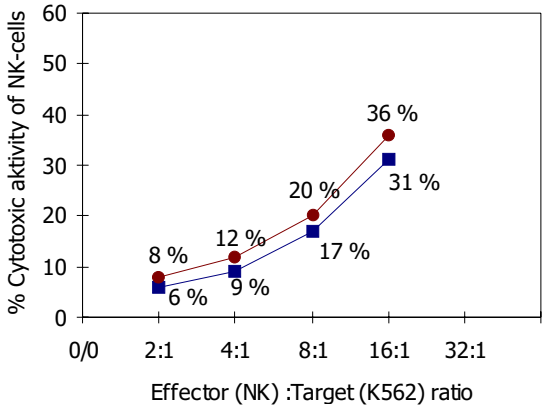

Fig. 5. The cytotoxic activity (y-axis) of the CD56 Multisort Kit NK-cells against the K562 (•) and HLA-G1 K562-transfectant cells (o), respectively, in 4 different efector to target cells ratios (x-axis).

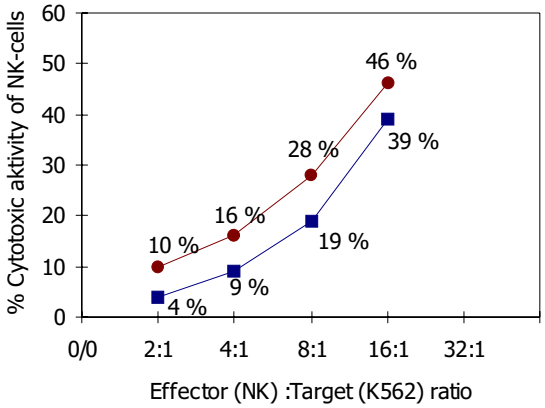

Fig. 4. The cytotoxic activity (y-axis) of the CD56 Multisort Kit NK-cells against K562 (•) and HLA-G1 K562-transfectant cells (o), respectively, in 4 different effector to target cells ratios (x-axis).

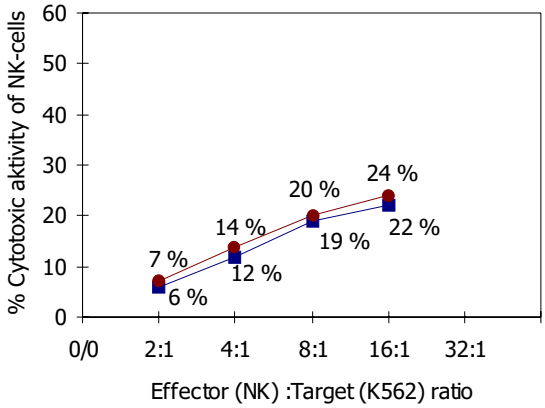

Fig. 6. The cytotoxic activity (y-axis) of the NK Cell Isolation Kit NK-cells against the K562 (•) and HLA-G1 K562-transfectant cells (o), respectively, in 4 different effector to target cells ratios (x-axis).

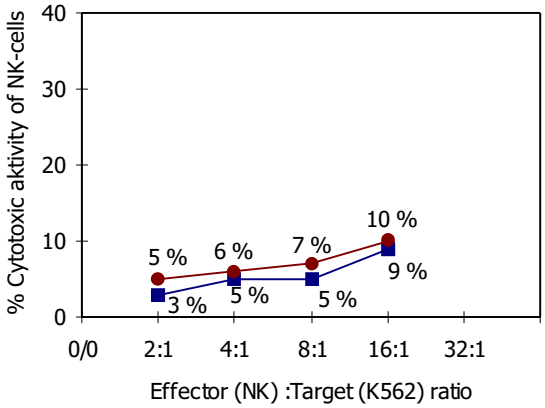

Fig. 7. The cytotoxic activity (y-axis) of one day old CD56 Multisort Kit NK-cells against the K562 (•) and HLA-G1 K562 transfected cells (o), respectively, in 4 different effector to target cells ratios (x-axis).

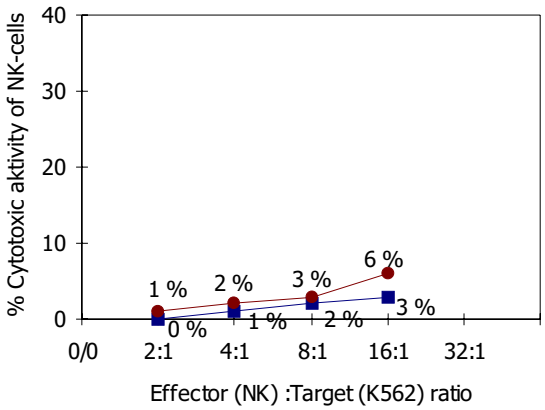

Fig. 8. The cytotoxic activity (y-axis) of one day old CD56 Multisort Kit NK-cells against the K562 (•) and HLA-G1 K562 transfected cells (o), respectively, in 4 different effector to target cells ratios (x-axis).

our preliminary results indicate that NK-cells are able to lyse both, the native K562 cells as well as HLA-G1-transfected K562 cells. Finally we can summarize that our results did not disclose any difference in NK-cell mediated cytotoxicity when K562 HLA-G1-transfectant cells were used as targets.

Discussion

The class Ia of HLA molecules are recognised by NK-cells either by inhibitory or stimulatory NK-receptors. When inhibitory signals prevail over the stimulatoryones, the target cells expressing the class Ia of HLA molecules are not lysed by NK-cells. Similarly, the class Ib of HLA molecules have been reported to induce the inhibitory signal in NK-cells, too (10, 11). As HLA-G and HLA-C are expressed at the foetal-maternal interface, in membranes of extravillous trofoblast cells, it is supposed that they are responsible for the protection of semiallogeneic foetus from decidual NK-cells during the pregnancy (2, 3, 4).

Cells of the human erythroleukemia K562 cell line express neither the class Ia nor the class Ib of HLA molecules, the fact of which results in their lysis when co-cultivated with NK-cells. One would therefore expect that should the K562 cells be transfected by HLA-G1 genes, they would resist their lysis, as their products would be recognised by NK-inhibitory receptors (KIR2DL4, CD94/NKG2A, ILT-2) (12–20).

Our results show that HLA-G-transfected K562 are lysed by NK cells, the fact of which is in contradiction with some reports (4, 6, 7, 8). The different results may be explained by different techniques used. NK-cells separated by the magnetic cell sorting system (MACS) were used in our experiments. The previous works were performed with peripheral mononuclear blood cells after their adherence on plastic, so NK-cells were not pure. Moreover, we used a europium release assay with incubation time of 100 minutes as opposed to the ^{51}chromium release assay with 4-hour incubation time. Also the europium is released out of target cells faster than in ^{51}chromium release (21). Although our results are in agreement with some other reports (9) they have to be considered as preliminary because of the small number of experiments done. More assays are needed as well as confirmations from other laboratories.

References

1. Carosella ED, Dausset J, Rouas-Freiss N. Immunotolerant functions of HLA-G. Cell Mol Life Sci 1999; 55 (3): 327—333.

2. Menier C, Riteau B, Dausset J, Carosella ED, Rouas-Freiss N. HLA-G truncated isoforms can substitute for HLA-G1 in fetal survival. Hum Immunol 2000; 61 (11): 1118—1125.

3. Onno M, Guillaudeux T, Amiot L, Renard I, Drenou B, Hirel B, Girr M, Semana G, Le Bouteiller P, Fauchet R. The HLA-G gene is expressed at a low mRNA level in different human cells and tissues. Hum Immunol 1994; 41 (1): 79—86.

4. Rouas-Freiss N, Marchal-Bras Gon•alves R, Menier C, Dausset J, Carosella ED. Direct evidence to support the role of HLA-G in protecting the fetus from maternal uterine natural killer cytolysis. Proc Natl Acad Sci USA 1997; 94: 11520—11525. **5. Tóth J, Buc M, Nikš M.** NK cell activity and association with the HLA class I antigen complex. Bratisl Lek Listy 1994; 95 (7): 323—331.

6. Rouas-Freiss N, Marchal RE, Kirszenbaum M, Dausset J, Carosella ED. The alpha1 domain of HLA-G1 and HLA-G2 inhibits cytotoxicity induced by natural killer cells: is HLA-G the public ligand for natural killer cell inhibitory receptors? Proc Natl Acad Sci USA 1997; 94 (10): 5249—5254.

7. Riteau B, Rouas-Freiss N, Menier C, Paul P, Dausset J, Carosella ED. HLA-G2, -G3, and -G4 Isoforms Expressed as Nonmature Cell Surface Glycoproteins Inhibit NK and Antigen-Specific CTL Cytolysis. J Immunol 2001, 166: 5018—5026.

8. Khalil-Daher I, Riteau B, Menier C, Sedlik C, Paul P, Dausset J, Carosella ED, Rouas-Freiss N. Role of HLA-G versus HLA-E on NK function: HLA-G is able to inhibit NK cytolysis by itself. J Reprod Immunol 1999; 43 (2): 175—182.

9. Weiss EH et al. Regulation von NK-Zellen und T-Lymphocyten durch HLA-Klasse Ib Molekule. Presentation-DGI 19.—21.9.2002 Kiel.

10. Allan D, Lepin E, Braud V, O'Callaghan C, McMichael A. Tetrameric complexes of HLA-E, HLA-F, and HLA-G. J Immunol Methods 2002; 268 (1): 43.

11. Buc M. Sprava z 1. medzinárodnej konferencie o HLA-G. Bratisl Lek Listy 1998; 99 (11): 629—632.

12. Faure M, Long EO. KIR2DL4 (CD158d), an NK cell-activating receptor with inhibitory potential: J Immunol 2002;168 (12): 6208—6214.

13. Rajagopalan S, Long EO. A human histocompatibility leukocyte antigen (HLA)-G-specific receptor expressed on all natural killer cells. J Exp Med 1999; 189 (7): 1093—1100.

14. Perez-Villar JJ, Melero I, Navarro F, Carretero M, Bellon T, Llano M, Colonna M, Geraghty DE, Lopez- Botet M. The CD94/NKG2-A inhibitory receptor complex is involved in natural killer cell-mediated recognition of cells expressing HLA-G1. J Immunol 1997; 158 (12): 5736—5743.

15. Pende D, Sivori S, Accame L, Pareti L, Falco M, Geraghty D, Le Bouteiller P, Moretta L, Moretta A. HLA-G recognition by human natural killer cells. Involvement of CD94 both as inhibitory and as activating receptor complex. Eur Jop Immunol 1997; 27 (8): 1875—1880.

16. Lee N, Llano M, Carretero M, Ishitani A, Navarro F, Lopez-Botet M, Geraghty DE. HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. Proc Natl Acad Sci USA 1998; 95 (9): 5199—5204.

17. Navarro F, Llano M, Bellon T, Colonna M, Geraghty DE, Lopez-Botet M. The ILT2 (LIR1) and CD94/NKG2A NK cell receptors respectively recognize HLA-G1 and HLA-E molecules co-expressed on target cells. Europ J Immunol 1999; 29 (1): 277—283.

18. Colonna M, Samaridis J, Cella M, Angman L, Allen RL, O'Callaghan CA, Dunbar R, Ogg GS, Cerundolo V, Rolink A. Human myelomonocytic cells express an inhibitory receptor for classical and nonclassical MHC class I molecules. J Immunol 1998; 160 (7): 3096—3100.

19. Colonna M, Navarro F, Bellon T, Llano M, Garcia P, Samaridis J, Angman L, Cella M, Lopez-Botet M. A Common Inhibitory Receptor for Major Histocompatibility Complex Class I Molecules on Human Lymphoid and Myelomonocytic Cells. J Exp Med 1997; 186: 1809—1818.

20. Lanier LL. Natural killer cells fertile with receptors for HLA-G? Proc Natl Acad Sci USA 1999; 96 (10): 5343—5345.

21. von Zons P, Crowley-Nowick P, Friberg D, Bell M, Koldovsky U, Whiteside TL. Comparison of europium and chromium release assays: cytotoxicity in healthy individuals and patients with cervical carcinoma. Clin Diagn Lab Immunol 1997; 4 (2): 202—207.

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