Transplant Int (1989) 2: 209-213

TRANSPLANT
International
© Springer-Verlag 1989

Lymphokine-activated killer (LAK) activity to cultured rat kidney parenchymal components in vitro

Y. Nietosvaara[1], R. Renkonen[1,2], P. Mattila[1], and P. Häyry[1]

[1] Transplantation Laboratory and
[2] Department of Bacteriology and Immunology, University of Helsinki, Haartmaninkatu 3, SF-00290 Helsinki, Finland

Abstract. The susceptibility of cultured rat kidney parenchymal components to natural killer (NK) cell and lymphokine-activated killer (LAK) cell-mediated lysis in a 4-h in vitro ^{51}chromium assay was investigated. Large granular lymphocytes (LGL) in the spleen and in the kidney allograft were able to lyse YAC cells during rejection, but they did not damage target endothelial, glomerular mesangial, glomerular epithelial, or tubular cells in resting state. Stimulation of the target cells with gamma-interferon - known to induce MHC (class II) antigens on the target cell surface - did not make the target cells susceptible to NK-mediated lysis. LAK cells generated by a 3-day incubation with interleukin-2 (IL-2) effectively lysed both YAC and P815 target cell lines. LAK cells were also slightly cytotoxic to all tested parenchymal target components in resting state. Gamma-interferon treatment of the cultured parenchymal cells prior to the chromium release assay, however, reduced LAK-mediated parenchymal cell cytotoxicity to nearly nondetectable levels. Obviously, many lymphokines, including IL-2 and gamma-interferon, are produced during rejection at the site of inflammation. This might induce the generation of LAK cells in situ as the lymphokines induce the production of MHC antigens in the graft. We interpret these findings as indicating that regardless of the generation of LAK, the protective effect of gamma-interferon neutralizes the LAK effect, and we suggest that neither LGL nor LAK cells play any essential role in rat kidney allograft rejection.

Key words: Kidney components, cultured, and lymphokine-activated killer activity - Lymphokine-activated killer activity, in the rat.

Offprint requests to: R. Renkonen

Large granular lymphocytes (LGL) are among the first lymphoid cells to appear in a rejecting rat kidney allograft [16]. The LGL increase rapidly in number, peak on day 3 after transplantation, and disappear shortly thereafter. The concomitant lymphoid blastogenic response reaches its maximum slightly later, on day 5 after transplantation, in drug-unmodified rat renal allograft rejection. Mature tissue macrophages and granulocytes appear even later, on day 6 and thereafter [16, 17, 22]. Little is known about how these different inflammatory cell types actually destroy the allograft.

Allospecific T cells are most likely essential for the induction and effector phase of allograft rejection. Particularly T helper (CD4), but also T killer (CD8), phenotypes have been shown to restore the ability of irradiated recipients to reject an allogeneic skin or organ graft [7, 13, 29]. However, these donor-specific lymphocytes represent only a small fraction (less than 1%) of graft-infiltrating cells [12, 19], indicating that other effector mechanisms might also exist.

T helper cells produce interleukin 2 (IL-2) in response to specific antigenic stimulation. The recruitment and activation of nonspecific cells by IL-2 inside the graft could act as one effector arm in allograft rejection. Lymphokine-activated killer (LAK) cells can be generated by culturing lymphocytes in the presence of IL-2 [4, 5, 30]. Some investigators provide evidence that precursors for these LAK cells are LGL [9, 25, 32]; others believe LAK cells can be generated from all T cells in general [2, 20]. Recent studies suggest that natural killer (NK) cells are activated by IL-2 alone, whereas peripheral resting T cells require the help of accessory cells as well [24, 26]. LAK cells display cytotoxicity against several tumor lines [4, 30]. Recently, it has been shown that normal tissue cells can also be lysed by LAK cells [15, 27]. In this study we have investigated

whether NK cells and/or LAK cells damage cultured renal cellular components in vitro.

Materials and methods

Isolation and culture of kidney components [14]

Kidneys were removed from 5-10-week-old DA (AgB4, $RT1^a$) rats and immediately put into cold phosphate-buffered saline (PBS). The kidney cortex was separated carefully from the medulla with a scalpel, washed twice with PBS, and pushed through a 250-μm steel mesh with a syringe. The minced tissue was diluted with serum-free, D-valine minimum essential medium (MEM; Gibco, Paisley, Scotland), supplemented with 50 μg/ml gentamycin (Gibco), 2 μmol/ml glutamine (Gibco), and 5 μmol/ml HEPES-buffer. Tubular components were isolated by washing the tissue through a 100-μm steel mesh; glomerular components were collected by filtrating the supernatant through a 75-μm steel mesh. The isolated components were washed twice with serum-free MEM.

After centrifugation, one part of the glomerular components was treated with type IV collagenase (Sigma, St. Louis, Mo), 750 IU/ml in 5 ml of serum-free MEM, at +37°C and 5% CO_2 for 30 min, shaken every 5 min. After collagenase treatment the components were washed with MEM containing 20% fetal calf serum (FCS; Sera-Lab, Sussex, UK) and centrifuged at 200 *g* for 5 min.

Different cells were cultured in T-25 flasks (Nuclon, Copenhagen, Denmark). For tubular and glomerular epithelial cells (tubular and untreated glomerular components, respectively), culture flasks and slides were coated with collagen and fibronectin (Sigma, St. Louis, Mo), both 10 μg/ml in PBS, for at least 1 h before culturing. The above-indicated medium with 10% FCS was used. For mesangial cells (enzyme-treated glomerular components), uncoated culture dishes and 20% FCS media were used instead.

For detachment of the cells from bottles, 5 ml versene (Gibco; 1:5000) was added. After incubation for 15 min at 37°C, the cells were washed through a 50-μm steel mesh to obtain a single cell suspension and then washed once in MEM.

Isolation and culture of endothelial cells

Four to 12-day-old DA rats were used. A modification of the Kasten method [10] was used. Rat hearts were minced with a scalpel and incubated three times in serum-free MEM with 0.2 mg/ml DNA:se (600 IU/mg, Sigma, St. Louis, Mo) and 0.2 mg/ml collagenase (183 IU/mg; Worthington). After the first incubation (10 min at 37°C, magnetic stirring) the supernatant was discarded. Two more 15-min incubations were performed and the supernatants were collected and filtrated through a 50-μm steel mesh. The cells were lysed with lysing reagent for 5 min at 37°C, followed by two washings. The single cells were diluted in MEM with 10% FCS and plated in T-25 tissue culture flasks or in Lab-Tek tissue culture chambers. After a 90-min incubation, the nonattached myocardial cells were discarded and the highly adherent endothelial cells were left in the flask. Endothelial cells usually grew to confluence in 5 days.

Characterization of cultured cell components

The purity and characterization of these cell components have been reported elsewhere [14]. In short, glomeruli and tubular fragments were separated using a combination of steel meshes with different pore sizes (100 and 75 μm, respectively). With this technique the glomeruli are practically free of capsules and vascular poles and there is very little tubular contamination in the glomerular fraction. Similarly, the tubular compartment contains very few glomeruli as judged by phase contrast microscopy. Trypan blue accumulates selectively only in the proximal tubuli as confirmed in histologic sections. In cell suspensions the glomerular fraction was totally free of stained tissue, whereas 75% of the tubular fragments were strongly trypan blue-positive, indicating that at least three-fourths of the tubular fraction contained proximal tubuli.

Endothelial cells formed a typical monolayer in confluence. Mesangial cells were fusiform, overlapping and forming "hills and valleys" in extended culture. Typical epithelial morphology was seen with phase contrast microscopy in glomerular epithelial cell and tubular cultures: cells were polygonal and formed a tightly packed, cobblestone-like monolayer in confluence.

Endothelial cells stained only with antivimentin (97%) and antifactor VIII (97%). Antidesmin stained mesangial cells (80%). Furthermore, 5%-10% of the cells in mesangial cell cultures stained positively with leukocyte common antibody (LCA), indicating that there was some white cell contamination or a crossreactive antigen in our mesangial cell population. No positive reaction with LCA was found in other cell populations. We were unable to make a clear distinction between glomerular epithelial cells and tubular cells with anticytokeratin and antivimentin. Similarly, antibrush border antibody and anti-Tamm-Horsfall antibody showed positive reactions in both populations.

Gamma-interferon stimulation

Isolated renal components were stimulated in the culture vehicles with 100 IU/ml rat gamma-interferon (a gift from Dr. P.H. v.d. Meide, Rijswijk, The Netherlands) added to the culture medium for 24 h.

Maintenance of tumor cell lines

The cell lines YAC-1 (sensitive to NK cell-mediated lysis) and cell line P815 (NK-resistant but LAK-sensitive) were maintained in continuous cell cultures in RPMI supplemented with antibiotics and 10% FCS.

Preparation of effector cells

Spleens from normal 8-12-week-old DA rats were removed, immediately placed in cold culture media (RPMI 1640 medium; Gibco) supplemented with 10% FCS (Sera-Lab) and 50 μg/ml gentamycin (Gibco), and pushed through a 250-μm steel mesh with a scalpel. Red cells were lysed with lysing reagent for 5 min at 37°C and washed twice; the remaining splenocytes were incubated in nylon wool columns for 1 h at 37°C. Nylon wool-passed cells were fed with iron powder and the phagocytic monocytes were removed with a magnet after a 45-min incubation. The remaining cells - mainly T cells and LGL - were cultured on plastic Petri dishes in the presence of 500 IU/ml IL-2 (a gift from T. Juhani Linna, Du Pont, Glenolden, Pa) in a humidified chamber at 37°C, 5% CO_2. After 3 days of incubation, the loose cells were collected and the remaining adherent cells were removed mechanically from the culture dishes with a rubber policeman [31]. For cytotoxic assays the nonadherent and adherent cell populations were used separately.

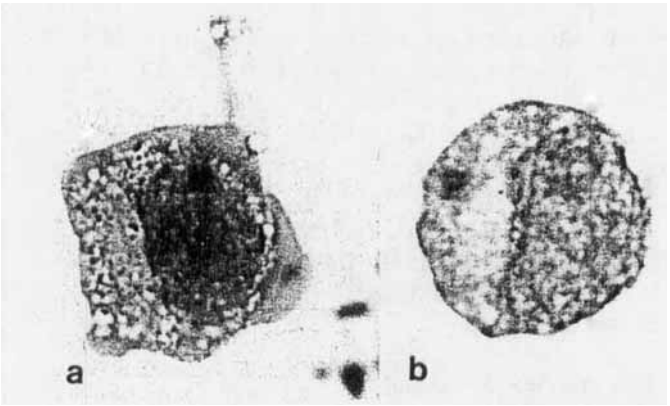

Fig. 1. a *LGL-LAK:* In MGG-stained cytocentrifuge smears, about 80% of the adherent cells were large LGL blasts with strongly basophilic cytoplasm and abundant azurophilic granules. b *non-LGL-LAK:* Approximately 80% of the nonadherent cells displayed similar characteristics without azurophilic granules and were identified as lymphoid blasts

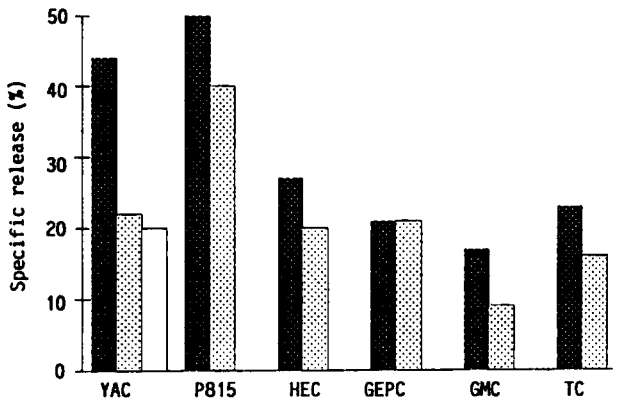

Fig. 2. LAK-mediated lysis of resting parenchymal components in a 4-h ^{51}chromium release assay. Specific release is presented as E:T ratio 100:1. Each column represents a mean of triplicate samples. One representative experiment is displayed. *YAC* and *P815*, tumor cell lines; *HEC*, heart endothelial cells; *GEPC*, glomerular epithelial cells; *GMC*, glomerular mesangial cells; *TC*, tubular cells. ▩, LGL-LAK; □, non-LGL-LAK; □, normal spleen cells

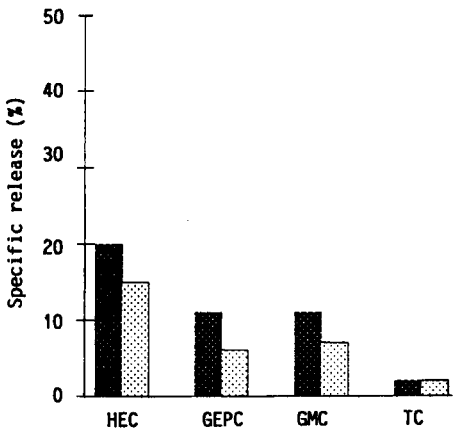

Fig. 3. LAK-mediated lysis of different parenchymal target cells treated with gamma-interferon (100 IU/ml) for 24 h. ▩, LGL-LAK; □, non-LGL-LAK

Cytotoxic assay

Target cells were plated in a concentration of 2000 cells in 100 µl culture media per microwell on microtiter plates (Nunc, Roskilde, Denmark). One hundred µl culture media containing ^{51}Cr was added and 24 h later the target cells were washed three times with warm culture media. Effector cells were then plated on the targets in different concentrations (100:1, 50:1, 25:1, and 12:1) in groups of three and incubated for 4 h. Subsequently, 100 µl of supernatant was collected from each microwell and the radioactivity in the sample was measured in a gamma counter (Wallac, Turku, Helsinki, Finland). The maximum release of chromium was obtained by lysing the labeled targets with 1% solution of Triton X-100 and spontaneous release by adding only culture media on the target cells. The percentage of specific release was calculated using the formula: [(cpm test sample - cpm control): (cpm maximum release - cpm control)] × 100.

Results

Nylon wool-passed splenocytes were stimulated with IL-2 on plastic Petri dishes. After 3 days of incubation, the majority of cells were floating loose in the media. The nonadherent cells were collected separately from the adherent cells, the latter being removed from the plastic Petri dishes by a rubber policeman. In MGG-stained cytocentrifuge smears, about 80% of the adherent cells were large (approximate diameter 20 µm), LGL-blasts with strongly basophilic cytoplasm and abundant azurophilic granules (Fig. 1a). Approximately 80% of the nonadherent cells displayed similar characteristics without azurophilic granules and were identified as lymphoid (non-LGL) blasts (Fig. 1b).

After stimulation with IL-2 for 3 days, both adherent (LGL) and nonadherent (non-LGL) cell populations effectively lysed YAC and P815 target cell lines; yet, adherent cells killed YAC more effectively than nonadherent cells did. Normal spleen cells lysed only YAC and not P815 cells. These functional properties are typical of LAK and NK activity, respectively.

Both IL-2-stimulated cell populations expressed cytotoxicity to cultured kidney components in a 4-h cytotoxic assay (Fig. 2), while normal spleen cells did not kill any of these targets. Renal parenchymal components were less susceptible to LAK-mediated lysis than YAC and P815 cells; the specific release was between 15% and 30% for the kidney targets and 40%-60% for the tumor cells with an effector: target ratio of 100:1. In individual experiments no significant differences were noted among the four different kidney components in their susceptibility to LAK cell-mediated lysis. The adherent LGL-LAK were slightly more effective (P= NS) than the nonadherent non-LGL-LAK in all of our experiments. Gamma-interferon treatment of target cells for 24 h

reduced LAK-induced lysis to nearly nondetectable levels (Fig. 3). The reduction was equal in all target populations.

Discussion

LAK cells display a wide range of cytotoxicity to different malignant cell lines and are able to lyse some NK-resistant tumor cells [4, 5, 30]. Recently, it has been reported that normal lymphocytes and endothelial cells are also vulnerable to LAK-mediated lysis in vitro [15, 27]. In our experiments LAK cells damaged all tested cultured rat kidney parenchymal components (heart endothelial, glomerular epithelial, mesangial and tubular components) in the ^{51}Cr assay. Our results are in accordance with these recent reports and suggest that parenchymal cells in general might be susceptible to LAK cell lysis.

A 24-h gamma-interferon stimulation of our various renal target components reduced LAK cytotoxicity to a nearly nondetectable level. Gamma-interferon has also been shown to protect human endothelial cells from LAK-mediated lysis [23]. However, gamma-interferon treatment did not protect tumor cell lines (K562 and Raji) against LAK cells. Gamma-interferon is known to enhance class I and II expression of these target cells [1, 33]. In many, but not all, cases NK activity is inversely correlated with class I expression [21, 27, 28]. Monoclonal antibodies to both class I and class II were ineffective in abolishing the protective effect of gamma-interferon when human endothelial cells were used as targets [23]. An antibody directed against gamma-interferon did, however, abolish the protection. Cold target inhibition studies show that the gamma-interferon effect does not alter the recognition but rather the effector phase of LAK cell killing [23]. Other cytokines possibly secreted in situ in a renal allograft (IL-1, IL-2 and TNF) were not effective in protecting human endothelium against LAK activity.

There is quite clear evidence that IL-2 is produced in large quantities in situ in a rejecting allograft [3, 6, 12, 29]. The generation of IL-2 is a prerequisite for the upregulation of inflammation; it is also a prerequisite for generating specifically cytotoxic cells to the allograft. IL-2 may also generate LAK activity against graft parenchymal cells. However, gamma-interferon is produced locally as well in the rejection [11]. This lymphokine, on the other hand, seems to protect the parenchymal cells from LAK-mediated damage. The results therefore suggest that the LGL or LAK cells in the allograft are not an essential effector component of rejection. This interpretation is in accordance with results demonstrating only a minor prolongation of graft survival in recipients receiving anti-Asialo GM1 (an antibody directed against NK cells) prior to transplantation [8]. Similarly thymectomized, total body-irradiated, and bone marrow-reconstituted "B rats" were not able to reject their kidney allografts, although cytotoxic LGL against YAC were recovered from these allografts on day 5 post-transplantation in quantities similar to those in rejecting normal controls [18].

Acknowledgements. We thank J. C. Hiserodt, M. D. for his personal advice concerning preparation of LAK cells. This work was supported by grants from the Academy of Finland, Helsinki; the Sigrid Juselius Foundation, Helsinki; the Kidney Foundation, Helsinki; the Finnish Cancer Foundation, Helsinki; the Farmos Research and Scientific Foundation, Turku; the Emil Aaltonen Foundation, Tampere; and the Duodecim Society, Helsinki, Finland.

References

1. Collins T, Korman AJ, Wake CT, Gimbrone MA, Strominger JL (1984) Immune-interferon activated multiple class II major histocompatibility complex genes and the associated invariant chain gene in human endothelial cells and dermal fibroblasts. Proc Natl Acad Sci USA 81: 4917-4921
2. Damle N, Doyle L, Bradley E (1986) Interleukin 2-activated human killer cells are derived from phenotypically heterogenous precursors. J Immunol 137: 2814-2822
3. Forbes RDC, Gomersall M, Lowry RP, Blackburn J (1984) Cellular mechanisms of cardiac allograft rejection. Heart Transplant 4: 300-311
4. Grimm EA, Muzumder A, Zhang H, Rosenberg SA (1982) Lymphokine activated killer cell phenomenon. Lysis of natural killer cell-resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes. J Exp Med 155: 1823-1841
5. Grimm EA, Robb FJ, Neckers LM, Lachman LB, Wilson DJ, Rosenberg SA (1983) Lymphokine activated killer cell phenomenon. III. Evidence that II-2 is sufficient for direct activation of peripheral blood lymphocytes into lymphokine activated killer cells. J Exp Med 158: 1356-1361
6. Gurley KE, Lowry RP, Forbes RDC (1983) Immune mechanisms in organ allograft rejection. II. T helper cells, delayed-type hypersensitivity, and rejection of renal allografts. Transplantation 36: 401-405
7. Hall BM, Dorsch SE (1984) Cells mediating allograft rejection. Immunol Rev 77: 31-59
8. Heidecke CD, Araujo JI, Kupiec-Weglinski JW, Fiers W, Ault K (1985) Lack of evidence for an active role for natural killer cells in acute rejection of organ allografts. Transplantation 40: 441-447
9. Itoh K, Tilden A, Kumagai K, Balch C (1985) Leu-11$^+$ lymphocytes with natural killer (NK) activity are precursors of recombinant interleukin 2 (rIL 2)-induced activated killer (LAK) cells. J Immunol 134: 802-807
10. Kasten FH (1972) Rat myocardial cells in vitro: mitosis and differentiated properties. In Vitro 8: 3-9
11. Lowry RP, Blais D, Harcus D, Hood L (1989) Studies on cytokine transcription and release by infiltrating rejecting rat/mouse heart grafts in normal and tolerant hosts. Transplant Proc: in press

12. Manca F, Ferry B, Jaakkola M, Halttunen J, Horsmanheimo L, Häyry P (1987) Frequency and functional characterization of specific T-helper cells infiltrating rat kidney allografts during acute rejection. Scand J Immunol 25: 255-261
13. Mason DW, Callman MJ, Arthur RP, Morris PJ (1984) Mechanisms of allograft rejection: the roles of cytotoxic T-cells and delayed-type hypersensitivity. Immunol Rev 77: 167-184
14. Mattila P, Nietosvaara Y, Ustinov J, Renkonen R, Häyry P (1989) Conditions of culture and regulation of antigen expression in different parenchymal cell types of rat kidney and heart. Kidney Int 36: 228-233
15. Miltenburg AMM, Meijer-Paape ME, Daha MR, Paul LC (1987) Endothelial cell lysis induced by lymphokine-activated human peripheral blood mononuclear cells. Eur J Immunol 17: 1383-1386
16. Nemlander A, Saksela E, Häyry P (1983) Are "natural killer" cells involved in allograft rejection? Eur J Immunol 13: 348-350
17. Nemlander A, Soots A, Häyry P (1984) In situ effector pathways of allograft destruction. I. Generation on the "cellular" effector response in the graft and the graft recipient. Cell Immunol 89: 409-419
18. Nemlander A, Leszczynski D, Halttunen J, Renkonen R, Soots A, Häyry P (1987) Evidence that thymectomized, bone marrow reconstituted rats do not reject their allografts. Transplantation 44: 66-70
19. Orosz CG, Zinn EN, Sirinek LP, Ferguson RM (1986) In vivo mechanisms of alloreactivity. I. Frequency of donor-reactive cytotoxic T-lymphocytes in sponge matrix allografts. Transplantation 41: 75-83
20. Phillips JH, Lanier LL (1986) Dissection of the lymphokine-activated killer phenomenon. Relative contribution of peripheral blood natural killer cells and T lymphocytes to cytolysis. J Exp Med 164: 814-825
21. Quillet A, Presse F, Marchiol-Fournigault C, Harel-Bellan A, Benbudunan M, Ploegh H, Fradelizi D (1988) Increased resistance to non-MHC-restricted cytotoxicity related to HLA A, B expression. J Immunol 141: 17-20
22. Renkonen R, Soots A, Willebrand E von, Häyry P (1983) Lymphoid cell subclasses in rejecting renal allograft in the rat. Cell Immunol 77: 187-195
23. Renkonen R, Ristimäki A, Häyry P (1988) Gamma-interferon protects human endothelial cells from LAK-mediated lysis. Eur J Immunol 18: 1839-1842
24. Roosnek EE, Brouwer MC, Kipp JB, Aarden LA (1986) Monocyte-dependent induction of proliferation of human peripheral T cells by recombinant interleukin 2. Eur J Immunol 16: 35-40
25. Salup RR, Mathieson BJ, Wiltrout RH (1987) Precursor phenotype of lymphokine-activated killer cells in the mouse. J Immunol 138: 3635-3639
26. Silvennoinen O, Vakkila J, Hurme M (1988) Accessory cells, dendritic cells or monocytes, are required for the LAK cell induction from resting T cell but not from NK cell precursors. J Immunol 141:1404-1409
27. Sondel PM, Hank JA, Kohler PC, Chen BP, Minkoff DZ, Molenda JA (1986) Destruction of autologous human lymphocytes by interleukin 2-activated cytotoxic cells. J Immunol 137: 502-511
28. Stam NJ, Kast WM, Voordouw AC, Pastoors LB, Hoeven F van der, Melief CJM, Ploegh HL (1989) Lack of correlation between levels of MHC class I antigen and susceptibility to lysis of small cellular lung carcinoma by natural killer cells. J Immunol 142: 4113-4117
29. Tilney NL, Kupiec-Weglinski JW, Heidecke CD, Lear PA, Strom TB (1984) Mechanisms of rejection and prolongation of vascularized organ allografts. Immunol Rev 77: 185-216
30. Vujanovic NL, Herberman RB, Hiserodt JC (1988) Lymphokine-activated killer cells in rats: analysis of tissue and strain distribution, ontogeny, and target specificity. Cancer Res 48: 878-883
31. Vujanovic NL, Herberman RB, Maghazachi AA, Hiserodt JC (1988) Lymphokine-activated killer cells in rats. III. A simple method for the purification of large granular lymphocytes and their rapid expansion and conversion into lymphokine-activated killer cells. J Exp Med 167: 15-29
32. Vujanovic NL, Herberman FB, Olszowy MW (1988) Lymphokine-activated killer cells in rats: analysis of progenitor and effector cell phenotype and relationship of natural killer cells. Cancer Res 48: 884-889
33. Wong GHW, Clark-Lewis I, McKimm-Breschikin JL, Harris AW, Schrade JW (1983) Interferon-gamma induces enhanced expression of Ia and H-2 antigens on B lymphoid, macrophage and myeloid cell lines. J Immunol 131: 788-793