DOI: 10.21276/ijabpt.2016.7.4.18

INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

www.ijabpt.com ISSN : 0976-4550

Volume-7, Issue-4, Oct-Dec-2016 Coden IJABFP-CAS-USA Copyrights@2016

Received: 17th Aug 2016 *Revised: 30th Sept 2016* *Accepted: 3rd Oct 2016*

DOI: 10.21276/Ijabpt, http://dx.doi.org/10.21276/ijabpt *Research article*

DIFFERENTIAL ASSAYS USED IN STUDY OF NATURAL KILLER CELL CYTOTOXICITY ASSAY

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ABSTRACT: In the present study we examined the effect of ALG on important immunological function. Our results show that ALG enhances NK cell cytotoxicity by non-adherent PBMC in aplastic anemia patients. These preliminary observations indicate the ALG may be clinically good immunological reagent for correcting NK cell cytotoxicity *in vivo* during immune suppressive therapy. These results demonstrate some unique feature of ALG as on immunomodulator at cytokine receptions in NK cell from the PBMC of both healthy and aplastic patients. The ALG also has unique time sequence in its action since the *in vivo* responses are possible to demonstrate only of certain points.

Key words: ALG, NK cell cytotoxicity, Aplastic anemia, PBMC

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INTRODUCTION

NK cells are the effectors which mediate the natural immunity by virtue of their inherent ability of lyse transformed or viral infected cells in an MHC non restricted fashion (Locasciulli et al 2007). In human peripheral blood they represent 5-10 % of monocellular cells and are rare in other lymphoid organs (Risitano et al 2010). Phenotypically these are large granular lymphocytes human NK cells are predominantly CD16+ and CD56+ (Marsh et al 2006; 2014). The CD16 and CD56 markers exhibit restricted distribution in peripheral blood and serve as indicators of NK cells (Rosenfeld et al 1995; 2003). NK cells comprise an important effector population that has been implicated in surveillance against tumor metastases, virally infected host cells, suppression of numeral immune response and regulation of hematopoiesis (Camitta 2000).Cluster differentiation marker CD2 is expressed by T lymphocytes and NK cells. It function as receptor for lymphocyte function associated antigen-3 (LFA-3) which is widely distributed on endothelied cells, epithelial cells and most of the blood cells including erythrocytes (Frickhofen et al 2003; Young et al 2006). The interaction between CD2 on T-lymphocytes and LFA-3 on erythrocytes lead to the formation of rosettes (Teramura et al 2007). Certain CD2 specific antibodies inhibit T-lymphocyte function while other activates the T cells (Scheinberg et al 2011; 2012). CD2 molecule plays an important role in thymocyte differentiation. Human T cells have an affinity and bind spontaneously to sheep red blood cells (SRBC) through CD2 receptor on their surface. This binding can be visualized as rosette in which red cells are seen to cluster around a central lymphocyte.

METHODOLOGY

Principle of assay

The NK cell activity is estimated quantitatively by radioactive chromium release assay. In this assay viable target cells are labeled with radioactive sodium (51 Cr). This chromium is released in the supernatant when target cells are lethally acted upon by the cytotoxic effector cells. The radioactivity of chromium present in the supernatant is measured with Gamma Counter. This count gives direct estimate of lytic potential of effector cells (NK).

NK Cell Cytotoxicity assay

NK cell cytotoxicity was evaluated by standard 51 Cr release assay. Peripheral blood monocellularcells (PBMC) from normal healthy volunteers and aplastic anemia patients were separated and cell concentration was adjusted to $2x10^6$ cells/ml in CRPMI-1640. These cells were stimulated at 37^{o}C for 1 to 6 hrs. with human recombinant interferon gamma (IFN) gamma 100µ/ml) and antilymphocyte globulin ALG in various log of dilutions from Nest ALG, 1:100, 1:1000, 1:10000 per well 9Nest is 50µ lit in 450 µ lit PBMC $2x10^6$). After incubation cells were collected, counted and finally adjusted to $4x10^6$ cell/ml.

Radiolabelling of target cells and cytotoxicity assay

K562 cell line was used as target for NK cells. Approximately $1x10^6$ target cells were resuspended in 50µ lit of FCS and labeled with 100 uCi of 51 Cr (Sodium chromate in saline specific activity 50-150 mCi/mg BARC, Bombay, India) for 90 min at 37^{o}C. After incubation these cells were washed thrice, counted and finally adjusted to $1x10^5$ cells/ml in complete media. Target cells were added to 96 well U-bottom plate and incubated with effector cells in various E:T (effector target) 100:1, 50:1, 25:1 for maximum release triton-X (3% V/V) and for spontaneous release, complete media used instead of effector cells. The plate was centrifuged at 500 RPM for 5 min and kept at 37^{o}C for 4 hr. Thereafter, it was again centrifuged at 500 RPM for 5 min and 100µ lit of supernatant was harvested from each well. Each set was taken in triplicate. Supernatant was counted in multigamma counter. Percent cytotoxicity was calculated with mean CPM of triplicate for each group as follows.

Interleukin (IL-2) assay

IL-2 is exclusively produced by activated T-lymphocytes and by NK cells. Resting T cells do not produce IL-2. Upon activation IL-2 is released by cells within hours. IL-2 act on its target via another molecule, the IL-2 receptor (IL-2 r) which is expressed on T lymphocytes, a subset and some macrophases. IL-2R is not expressed by resting T cells.

Generation of IL-2

Briefly, 0.5×10^6 cells/ml of human PBMNC suspension was cultured in RPMI-1640 media for 48hr in the presence and absence of antilymphocyte globulin (ALG). Phytochemagglutinin (PHA) was used as positive control. Supernatants were harvested and stored at -70^{o}C.

Bioassay of IL-2 using Enzyme linked Immunosorbant Assay (ELISA)

IL-2 assay was carried out as solid phase enzyme immunoassay based on multiple antibody sandwich principle. The ELISA plate wells precoated with mouse monoclonal antibody specific for human IL-2 was used to capture IL-2 present in standard and unknown samples. An anti-IL-2 rabbit polyclonal antibody which binds to was added to each well followed by peroxide TMB substrate solution. The substrate initiated a peroxidase catalyzed colour change which was stopped within 5 min by acidification with stop solution; the absorbance measured by ELISA reader at 450 nm was proportional to concentration of IL-2 present in samples. A standard curve was obtained by plotting IL-2 standard concentrations in experimental samples were then determined from standard curve. Results are represented as concentration of IL-2 in pg/ml.

T-Cell Rosette Formation

In brief 1×10^6 cells/ml of human PBMNC suspension was incubated with various 10 g of dilutions from neat ALG: 1:100, 1.000 and 1.10000 at 37^{o}C for 1 hr. Later cells were washed and suspended in fetal calf serum. For T-cell rosette human PBMNC suspension in fetal calf serum was mixed with sheep red blood cells (SRBC) in saline to obtain SRBC and lymphocyte ratios of 10:1 and 1:1 for active and total T cell rosetts respectively. The tubes with cells were centrifuged gently at 200 g for 5 min resuspend and rosettes were counted immediately for active T cell rosetts. For total T cell rosette tubes were centrifuged and rosetts were counted after overnight incubation at 4^{o}C. Lymphocytes with > 3 SRBCS were counted as rosetts. A minimum of 300 lymphocytes were enumerated and results are express as percentage of total PBMNC.

Preservation of Lymphocytes of Aplastic anemia (AA) patients and Healthy Normal controls for flow Cytometry Analysis

NK cell phenotypes will be enumerated by flow cytometry using CD3/CD16+CD56+. We also have plans to enumerate other lymphocyte subpopulations like T and B cells using specific Moabs. This study will show us the effect of therapy of PBMC subsets and possibly the correlation with outcome of treatment with ALG.

Collection and storage of Lymphocyte Samples

PMBC have been obtained and stored from patients before starting of ALG therapy and 3 and 6 months after it. In 4 patients cells will be stained with fluorochrome conjugated antibodies in a flowcytometer. Viable lymphocytes were obtained from samples of whole blood of AA and healthy normal controls.Lymphocytes were stored in sterile culture medium at a concentration of 5×10^6 cells/ml in sterile freeze vials.Freezing medium: Dimethyl sulfoxide (DMSO), inactive fetal calf serum FCS (10% DMSO+90% FCS).

PROCEDURE

Cell suspension was centrifuged to obtain a pellet and discarded supernatant.The cells were suspended in FCS 90% with 10% DMSO in cold condition was used as a cryoprotective solution. The vials were appropriately labeled with the name, date and number of cells been stored.

Status of NK Cell phenotypes

Aplastic anemia (AA) is pancytopenia resulting from nonfunctioning bone marrow. It has been shown by various workers, that there is reduced number of natural killer (NK) cells in peripheral blood patients with AA low cytotoxic potential. Treatment with ALG has been shown to cause an up regulation of NK cell number in peripheral blood and NMK activity perhaps by renewal of bone marrow function. An increase in NK cell activity in with AA patients has been used as a marker for monitoring responses to anti-lymphocyte globulin (ALG). Taking a cue from these results we studied the NK cell activity in patients with AA before and after AKG is f interest in addition to its immunosuppressive action.

Mechanism of Augmentation of NK cell activity in Aplastic Anemia by ALG

Antilymphocyte globulin (ALG) is raised in horses by hyper immunizing them with thoracic duct lymphocytes. It thus contains high titers of antibodies against various lymphocyte surface antigens like TCR, CD3, CD4, CD8, CD16, CD56, IL-2R and surface lgs etc. Immediately after injection of ALG there could be some lymphocytopenea due to binding of these is antibodies to lymphocytes. After few weeks there is antibody production to ALG or ALG may be catabolized. This leads to low concentration of ALG which may be optimal in immune stimulation of T-cells and IL-2 production. This in turn may lead to a direct upregulation of NK cell activity to augment the NK cell activity by gradually restoring hematopoiesis due to removal of suppressor T cells.

RESULTS AND DISCUSSION

The total lymphocyte count was different in each of the twenty aplastic anemia patients. NK cell activity of Aplastic anemia as specific lysis was reduced compared to twenty six normal controls. The low NK cell activity in aplastic anemia did not correlate with the severity of the disease. Changes in NK cell activity significantly varied between patients of pre ALG and post ALG treatments.These results for the first time show a time and dose response of ALG to enhance augmentation of NK cell cytotoxicity in aplastic anemia patients fig 1.

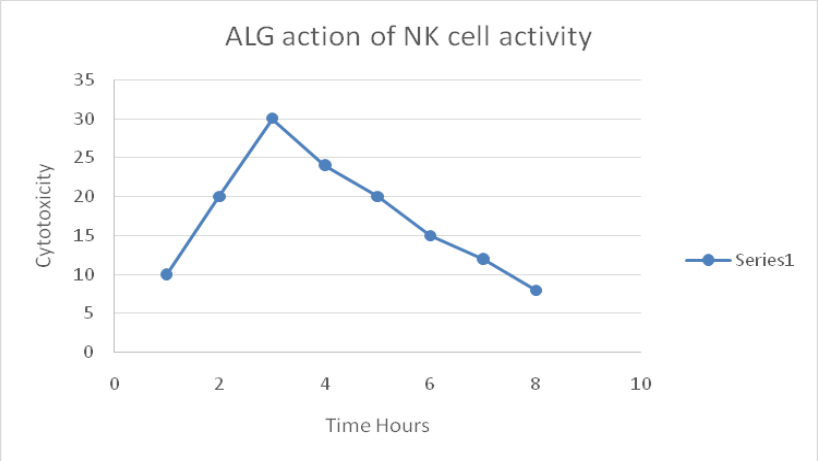

Fig 1: ALG action of NK cell activity

In all of twenty six normal controls and twenty patients examined ALG along increased the NK cell in dose response manner, in which 4 µg/ml for 1.1000 dilution from neat ALG induced maximum cytotoxicity.Further ALG action of NK cell activity *in vitro* with reference to time factor, cytotoxicity gradually increased up to 3rd hour of incubation of effector cell with ALG. Thereafter values of cytotoxicity returned to normal. Time and dose response factor was not much different both in normal controls from patients. The basal NK cell activity of aplastic anemia patients had greater reduction.

In the present study we examined the effect of ALG on important immunological function. Our results show that ALG enhances NK cell cytotoxicity by non-adherent PBMC in aplastic anemia patients. These preliminary observations indicate the ALG may be clinically good immunological reagent for correcting NK cell cytotoxicity *in vivo* during immune suppressive therapy. These results demonstrate some unique feature of ALG as on immunomodulator at cytokine receptions in NK cell from the PBMC of both healthy and aplastic patients. The ALG also has unique time sequence in its action since the *in vivo* responses are possible to demonstrate only of certain points.

Effect of ALG on T-cell Rosetting

To investigate the effect of ALG on T-cell resetting from healthy normal and aplastic anemia patients we studied total T-cell rosette which identifies total T-cells and the active stimulated or early T-cell rosetts which identifies a subpopulation of T-cells with receptors of high affinity for SRBC.ALG enhanced active T-cell resetting when PBMNC were exposed to 4 µg/ml or 1.1000 dilution from neat ALG. The increase was found to be statistically significant ($P<0.01$ or 0.02) from that of controls i.e. PBMNC without ALG treatment.Rosette formation really explained by the fact that the active T-cell rosette is performed under sub optimal concentrations for resetting (low SRBC per lymphocyte ratio and short incubation time between SRBC and lymphocytes).

Stimulation of IL-2 by ALG

Human PBMNC were stimulated with ALG *in vitro* and supernatants were collected after 48 hr and assayed for induction of IL-2 we have used sandwich ELISA for estimation of IL-2 induced. Our results suggests that ALG significantly stimulated IL-2 production ($P<0.01$ or 0.02).Results from number of studies have shown that the proliferation of T-lymphocytes during an immune response requires both the secretion of IL-2 and do novo expression of IL-2 receptor. In our study ALG has been found to stimulate T cell proliferation as well as IL-2 production significantly. Present data support a physiological role of ALG in modulating human lymphocyte activity through cytokine network.

CONCLUSION

We have studied NK cell cytotoxicity in twenty patients of aplastic anemia and compared it with that in 26 healthy controls. In the twenty patients and 26 controls, we have also studied the *invitro* effectof ALG on NK cell cytotoxicity at different time intervals and using different concentrations of ALG. NK cell cytotoxicity was assessed by chromium release assay using K562 cell line as target. Some of the patients we have studied, the NK cell cytotoxicity 3 and 6 months after onset of therapy.The basal NK cell activity was reduced in the patients when compared with the normal controls. In all of 26 normal and controls and twenty patients, *in vitro* increased the NK cell activity in a dose and time dependent manner. Maximum cytotoxicity was observed at 4 µg/ml or 1:1000 dilution of neat ALG.In the 4 patients, where NK cell cytotoxicity was also studied after therapy with ALG, it was observed that the NK cell cytotoxicity was higher after treatment with ALG in comparison to the pretreatment NK cell cytotoxicity.

Acknowledgements

We are very grateful to Dr.Naresh Tripati, Department of Immunology, Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGIMS), Lucknow, Uttar Pradesh for providing case reports of patients.

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ISSN : 0976-4550

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