IN VITRO MODULATION OF LYMPHOKINE - ACTIVATED KILLER (LAK) CELL FUNCTION BY AQUEOUS SUSPENSIONS OF INERT PARTICLES

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SUMMARY: Aqueous suspensions of inert particles were shown to inhibit the natural killer (NK) cell and lymphokine - activated killer (LAK) activity of peripheral blood mononuclear cells (PMBC) and large granular lymphocytes (LGL). This inhibition was induced with both latex and silica particles. The inhibition of cytotoxic cell function was not related to effector cell death as determined by trypan blue exclusion, but appeared to be more specific for NK cell function (45.6 ± 4.5 % suppression of baseline NK cell activity) than LAK cell function (10.3 ± 3.4 % supperssion of baseline LAK cell activity) when the effector cells were preincubated with latex particles for 4 hours ($p<0.05$). Greater inhibition of LAK activity (35.6 ± 5.4% suppression) could be obtained if the efector cells were incubated with the particles during the 72 hr IL - 2 incubation period. In all tests, silica particles appeared to be more potent in suppressing cytotoxic activity than did particles of latex. Glass adherent monocytes which were incubated with purified LGLs did not accentuate particle-induced suppression, suggesting that monocyte-mediated suppression was not a major mechanism in the induction of these abnormalities. These studies indicate that exposure to small particles may directly suppress NK cell and LAK cell function and could contribute to an impaired ability of the host to resist tumor growth.

Key Words: lymphokine, natural killer cells, lymphokine activated killer cells.

INTRODUCTION

Natural killer (NK) cells and lymphokine - activated killer (LAK) cells are important in the host resistance to neoplasma and viruses (6,9). These cytotoxic lymphocytes destroy existing tumor cells and prevent the formation of tumor metastases in mouse tumor models (7,19). The cytolytic activity of these antitumor cells is impaired in many patients with cancer, implying that the normal function of the NK cell and the NK - like LAK cell may be essential in prevention of tumor growth and metatases in humans as well as animals (17,23,29).

Interleukin - 2 (IL-2) is essential for the maintenance of cytotoxic cell function. IL-2 maintains and accentuates NK cell activity in culture (14). Prolonged incubation of lymphocytes with IL-2 induces the generation of a cytolytic lymphocyte with activity against a large number of NK - resistant tumor cells. This unique cytoxic cell is known as the LAK cell (6, 19). The NK cell and the LAK cell are large granular lymphocytes (LGLs), yet they appear to be distinct from each other and are characterized by definite functional and phenotypic differences (6,15).

Recently, it has been shown that aqueous suspensions of inert particles can inhibit NK cell activity (24). Importantly, injection of particles into experimental animals has resulted in profound in vivo inhibition of NK cell activity as well as an inability to reject tumor innocula (21). Since LAK cell may also be important in tumor rejection (15), the present study examined the simultaneous in vitro

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effects of particles on NK and LAK cell function. It was found that aqueous suspensions of particles inhibited both NK and LAK cell function, although the LAK cell appeared to be somewhat more resistant to this form of immunosuppression.

METHODS AND MATERIALS

Preparation of mononuclear cells suspensions

Peripheral blood mononuclear cells (PBMC) were isolated from whole heparinized blood by centrifugation on Ficoll-Hypaque gradients (4). The cells were washed 3 times in phosphate buffered saline (PBS) and resuspended in RPMI 1640 (Flow Laboratories, Hamden, CT) at $10x10^6$ cells/ml with penicillin (100 IU/ml) and streptomycin (100 μg/ml) The final cell suspensions contained 2-15 % monocytes by peroxidase staining (31) and approximately 1% neutrophils by Wright's stain.

A suspension of PBMC containing $5x10^7$ cells in 1 ml of human AB serum were incubated in nylon wool columns for 45 min at 37 degrees C. The nonadherent cells were eluted with 20 ml of warm medium. Any remaining B cells were detected by direct immunofluorescence with fluorescein-conjugated pepsin-digested anti- $F(ab')_2$ (Cappell Laboratories, Cochranville, PA) and were less than 3%. The number of monocytes was less than 1% as determined by both peroxidase staining and direct immunofluorescence exploying the monoclonal anti-monocyte antibody Leu M3 (Becton-Dickinson, Sunnyvale, Ca).

Percoll gradient centrifugation

Enrichment of large granular lymphocytes (LGLs) was accomplished as previously described (28). Briefly, to prepare density graduents for centrifugation, Percoll (Pharamacia Chemicals, Uppsala, Sweden) was mixed in various concentrations with medium and distiller water so that the final osmolar concentration was always 285 mosmol/liter at a pH of 7.4. Seven ml volumes of Percoll solutions were carefully layered into a 50 ml Falcon conical - bottomed test tubes starting with 45.0% Percoll (fraction 5), and grading by 2.5% concentration diminutions to 35.0% Percoll on the top (fraction 0). $100\text{-}250x10^6$ lymphocytes purified as described above were added and the tube was centrifuged at 550 x g for 45 min at 20 degrees C. The fractions were collected with a Pasteur pipett from the top and washed twice in medium. Recovery of the cells was 85% ±12 (SD) of the original input and the viability always exceeded 98%. The morphological analysis of the effector cell suspensions was determined with a Giemsa statin. Fractions 1 and 2 contained the largest amount of NK cell activity and morphologic LGLs and these two fractions were combined and used as the purified effector cells. These LGL fractions contained less than 1% monocytes by peroxidase staining, less than 1% monocytes by Leu M3 direct immunofluorescence, 83% LGLs by Giemsa stain, and 22.2% Leu 1 (Becton Dickinson) positive cells by direct immunofluorescence. In some experiments, glass adhernet cells consisting of 80% peroxidase staining mononuclear cells were prepared as previously described (22) and were added back to the purified LGL suspensions and incubated overnight before determining NK activity.

The IL-2 dependent generation of NK and LAK cell activity

The induction of the LAK cell has been previously reported (11). Briefly, LGLs were recovered from peripheral blood as described earlier. These cells were resuspended at $1x10^6$ cells/ml in RPMI 1640 medium with 10% HABS or 10% FBS (Flow laboratories) with penicillin and streptomycin and 0,10,30 or 100 units of IL-2 (Electro-Nucleonics, Silver Spring, MD) for 0 to 5 days. Dead effector cells were removed by density centrifugation on fetal bovine serum so that the final cell suspensions always contained greater than 98% viable cells. LAK cell function was then determined against the NK-resistant Daudi cell line (11). Maximum LAK cell activity was obtained at three days of preincubation with IL-2 (10 units/ml) as previously reported (11). Subsequently, all LAK CELL determinations were made using these same incubation parameters.

The effects of particles composed of polystryrene latex (0.1, 1.1 and 15.8 μ diameter, Sigma) and silica (.014 and 10 μ diameter, Sigma) on effector cell function were determined by preincubation of the particles with the effector cells for 0,2,8 or 16 hours prior to determination of NK cell function. In these cases the particles were washed threee times in PBS and resuspended in RPMI 1640 medium prior to the addition to the cultures.

Chromium release assay for NK cell activity

K562 (for the NK assay) and Daudi (for the LAK assay) cells were labelled by incubation of $5x10^6$ cells with 0.1 ml $Na_2\ ^{51}Cro_4$

Table 1: Effect of Latex Particles On NK and LAK Cell Function.

	Incubation time		
	0 hr	2 hr	16 hr
Particle Diameter**	NK CELL ACTIVITY*		
15.0	49.2±4.5	42.5±3.2	26.9±4.3
1.0	45.5±3.5	40.2±2.4	27.5±3.4
0.1	47.3±3.6	41.6±3.7	29.0±3.8
no particles	48.2±5.6	49.2±2.6	45.7±3.9
LAK CELL ACTIVITY*			
15.0	54.4±4.8	52.3±4.3	41.7±4.2
1.0	52.0±3.2	50.1±4.5	42.2±2.6
0.1	54.7±3.2	49.1±3.9	42.2±2.1
no particles	53.6±4.5	54.2±3.2	49.6±3.1

* Lytic activity of PBMC before removal of glass wool adherent cells is expressed as lytic units/10^7 effectors; n=3; mean±SD. NK activity was measured with fresh PBMC against the K562 target while LAK activity against Daudi targets was measured with PBMC which had been incubated with IL-2 (10 IU) for 72 hrs 37 degrees C.

** Particle diameter is expressed in microns. All effector cells were incubated with particles at a concentration of 0.5 mg/ml.

(specific activity of 1.0 mCi/ml New England Nuclear, Boston MA) in 0.2 ml of fetal calf serum for 45 min at 37 degrees C, followed by 3 washings with media. Ten thousand target cells were mixed with various effector cells suspensions to achieve effector target (E:T) ratios from 3:1 to 100:1 in sterile round bottom microtitier plates (Linbere; flow Laboratories, Hamden, CT). Each well contained final volume of 0.2 ml. The plate were incubated for 4 hr at 37 degrees C in humidified air plus 5% CO_2. After incubation the plates were centrifuged at 100 x g for 10 min and 0.1 ml of supernatant was carefullly removed. Lyzerglobin (Baker Chemical Co., Bethlehem, PA) was added to a series of wells to determine the maximum chromium release. Spontaneous release was defined by a series of wells with no effector cells. All samples were run in sextuplet. The percent NK or LAK cell activity was calculated as follows:

% specific cytotoxicity= 100 x experimental ^{51}Cr release-Spontaneous ^{51}Cr release maximum ^{51}Cr release-spontaneous ^{51}Cr release

Results were quantitated at various E:T ratios by determining the number of lytic units (LU) per 10^7 cells at a specific cytotoxicity of 30%.

Table 2: Effects of Particle Concentration on NK and LAK Cell Activity.

Particle Concentration (μg/ml)***				
	500	50	5	0.5
Particle size**		RELATIVE NK CELL ACTIVITY (%)*		
15	45.2	61.0	87.0	91.8
1.0	35.0	58.0	78.6	100.0
0.1	40.2	60.1	69.4	75.3
RELATIVE LAK CELL ACTIVITY (%)*				
15	80.3	89.0	95.4	93.0
1.0	76.9	82.7	91.2	97.3
0.1	76.5	84.6	92.3	99.2

* Relative lytic activity=100 x $\frac{\text{NK or LAK activity with particles}}{\text{NK activity with medium}}$

** Particle diameter is expressed in microns. Incubation time was 16 hours; n=3.

*** Latex particle concentration is always expressed in μg/ml, but can be converted to the number of particles/ml using the following formula: Concentration (particles/ml)= Concentration (μg/ml) (1.2 x 10^6 μg/(m^2x10^{-6})x1.33 x 3.14 x (radius in m^3).

RESULTS

Effects of latex particles on NK and LAK cell function

Polystyrene latex particles of various diameters were used as standard particles. The diameters of these spheroids were uniform in diameter and, thus, allowed more accurate determinations of the effects of particle size on immune function. Importantly, latex is not toxic to mononuclear cells. Latex particles (0.1, 1.0 and 15.0 μm in diameter) were incubated with PBMC for 0,2 and 16 hours after which NK and LAK cell activities were quantitated as described in the Methods. For NK activity PBMC containing glass adherent cells were used immediately after purification while LAK Cell activity was assessed with PBMC which had been incubated for 72 hrs with IL-2 (10 IU/ml). As can be seen in Table 1, NK and LAK cell activity were both impaired with all diameters of latex particles. (15, 1, and 0.1 μm) relative to the control PBMC not exposed to the latex particle ($P<0.05$). When particles were added to the effector cells without any incubation period, suppression of NK and LAK activity was noted, suggesting that preincubation was necessary for the inhibitory effect. Table I;I demonstrates the dose - response of particles to the cytotoxic cell function with the maximal suppression occuring in the 500 to 5 ug/ml concentration range. It is apparent from these findings that the LAK cell was relatively more resistant than the NK cell to the suppressive effects of particles.

Table 3: The effect of Monocytes on Latex Particle Inhibition of LAK Activity.

Particle diameter*	LAK Cell Activity[1]	
	Control suspension	Monocyte-depleted
10.0	35.4±3.3	65.0±4.3
1.0	30.2±4.7	48.3±4.4
0.1	33.8±3.2	50.7±2.5
no particles	44.3±6.8	70.0±3.2

* Particle diameter is expressed in microns. The particle concentration was 0.5 mg/ml.

[1] LAK activity was expressed as LU/10 cells; n=3, mean±SD.

Table 4: The Effect of Latex Particles on the LAK Activity of Purified LGLs.

	LAK Cell Activity (LU/10^7 Cells			
Particle diameter	Particle	concentration (ug/ml)		
	0.0	500	50	5
10.0	53.9±3.4	47.4±4.3	49.0±2.2	53.5±3.2
1.0	55.6±6.4	34.8±4.3	43.2±4.4	47.3±4.9
0.1	54.2±3.6	36.8±5.3	44.8±3.7	48.2±2.3

* Particle diameter is expressed in microns. The particle concentration was 0.5 mg/ml, n=3; mean±SD.

Effects of monocytes

The presence of monocytes can be associated with particle-induced suppression of NK activity (24). Indeed, suppressor monocytes have been previously described and may be important in regulation of cell-mediated immunity (22). To determine if the inhibition of LAK cell activity were also secondary to the presence of monocytes in the PBMC suspensions, adherent cells were removed with nylon columns. The resultant suspension

Table 5: The Effect of Particles of Different Composition on LAK Activity.

Particle*	LAK Cell Activity **
Latex (1.0 μm)	41.4±5.2
Latex (0.1 μm)	38.3±3.21
Silica (0.5 μm)	36.4±1.1
Silica (0.01 μm)	14.4±0.5
Control with no particles	52.5±1.5

* Particle size is expressed in microns. Particle concentration was 50 μg/ml.

** LAK activity is expressed in LU/10^7 effector cells as measured against the Daudi target. These were purified LGLs without monocytes; n=3; mean±SD.

contained less than 1% monocytes as determined by both peroxidase staining and direct immunofluorescenco employing the anti-monocyte monoclonal antibody Leu M3. As can be seen in Table 3, removal of monocytes did prevent suppression of LAK cell activity induced by the larger (15 μm) particles, but did not significantly influence the ihnibition associated with the smaller (1 and 0.1 mm particles), indicating that the observed suppression of LAK cell activity by particles might be dependent of particle diameter.

It has been demonstrated the particle exposure can inhibit the NK cell activity of LGLs (24). In order to determine if particles could directly inhibit the LAK activity of purified effector cells without monocytes, LGLs were enriched by Percoll gradient centrifugation. These suspensions contained less than 1% peroxidase-positive cells and less than 1% monocytes as defined by direct immunofluorescence with the anti-monocyte monoclonal antibody Leu M3. The effect of particles on the LAK cell function of LGLs is shown in Table 4. As can be seen, there was significant suppression of LAK cell activity at all particle concentrations ($p<0.05$).

To further exclude the possibility of monocyte-induced suppression, glass-adherent cells consisting of grater than 80% monocytes were added back into cultures of purified LGLs. The LGLs were incubated with latex particles (1.0 micron diameter, 50 μg/ml) for 72 hours in the presence of IL-2 as described earlier. The activity against the Daudi target at the end of incubation was 32.2 ± 2.2 (LU/1010^7 cells) in the presence of the particles and 46.9 + 4.3 (LU/10^7 cells) media without the particles (n=4, $p<0.05$). Add back of 2.5, 5, 10, 25 and 50% glass adherent cells (78.5 % monocytes by peroxi dase staining) to the purified LGLs resulted in respectively 3.2 ± 1.1, 4.2 ± 2.0, 4.9 ± 3.6, 2.2 ± 3.4 and 2.8 ± 8.6% inhibition of NK cell activity without the presence of latex particles. When 1.0 μm particles were added, there was no observed increase in the inhibition induced by the monocyte suspensions ($p>.01$). These results suggest that inert particles can inhibit the LAK cell activity of suspensions consisting of purified LGLs which are devoid of potentially immunosuppressive monocytes. Although monocytes may exert some suppressive effect on LGLs, the suppressive effects induced by suspensions of aqueous particles appear to be largely independent of monocyte induced suppression.

Suppression of NK and LAK cell activity by other forms of particles

To determine if the observed immunosuppression of LAK function were a trivial toxic property intrinsic to the latex bead and not inherent in other particles, particles of different composition and size were examined in the same assay. Silica (0.5 and 0.01 μm) and latex beads (1.0 and 0.1 μm) were studied in the same system. As can be seen in Table 5, all of these particles resulted in significant inhibition of NK cell function. This inhibition was comparable to that found with latex beads and did not result in lymphocyte death. However, the silica particles of 0.01 μm in diameter were extremely suppressive relative to the larger latex and silica particles.

DISCUSSION

NK cell and LAK cells may be important in tumor rejection and factors which suppress their cytolytic function may contribute to uncontrolled tumor growth (6,7,9, 14,15,19). Recently, there is some evidence that particle exposure may increase the probability of developing certain tumors (1,30). This is especially true in the case of silica particles which can induce cancers in both man and animals (5). This hypothesis has been confirmed in a recent animal study were silica exposure resulted in the profound depression of NK cell function and the inability to reject tumor innocula (21). A recent report has demonstrated that similar in vitro exposure of human LGLs to small particles can result in inhibition of human NK cell activity through monocyte-dependent and independent pathways (24). The present study has extended these observations and has demonstrated that aqueous suspensions of small particles can inhibit not only NK cells, but other cytotoxic cells as well.

In particular, the present study demonstrated that inert latex and silica particles resulted in profound depression of the LAK cell and NK cell activity of human PBMC. The suppression appeared to be time and dose dependent and was only partially related to the presence of monocytes in the mononuclear cell suspensions. These data

were further supported by the finding that purified suspensions of LGLs, the reputed effector cells of LAK activity, were directly inhibited by particle exposure. Add-back of monocytes to purified cultures of LGLs exposed to 1.0 μm latex particles did not enhance the observed inhibition, suggesting that monocyte-mediated suppression of LGL, LAK and NK cell activity was not a major mechanism.

The present study revealed that the degree of NK and LAK cell suppression appeared to be dependent on the diameter of the particles with greater suppression occurring with particles less than 15 um in dimater (Table I;I). The inhibition induced by larger particles was reversed by removal of glass-wool adherent cells. These results suggest that suppressor monocytes may have some role in the induction of some particle induced suppression as has been reported in other systems. Evidence from other studies have shown that particle size is crucial for the specific-biological effects induced on target cells by particle exposure, particularly when monocytes and macrophages have been exposed to particles (8). This phenomenon may be responsible for many of the observed effects relating to particle size in the present study.

The present study also suggested that LAK cells, although inhibited by both latex and silica particles, were more resistant to the suppressive effects of particles than were NK cells from the same individual. LAK cells are known to be "activated cells" and, thus, are more resistant to the effects of prostaglandins, corticosteriods and other immunomodulatory agents (12, 25). The results of the present study suggest that the same sort of resistance of LAK cells exist toward the suppressive effects of particle exposure.

The results of the present study demonstrated that aqueous suspensions of particles can directly down-modulate both NK and LAK cell activity. These results are supported by recent in vivo experiments in which mice received injections of silica particles (21). In previous study particles resulted in both a markedly impaired NK cell activity and a reduced ability of the host animal to reject tumor innocula. The suppression of NK activity and the degree of tumor load appeared to increase with the interval subsequent to particle exposure. These data provide valuable in vivo support for the results of the present study and suggest that these observations may be of considerable importance.

Occupational exposure to particles has been related to the development of both inflammatory and malignant disorders (16). The association of particles in the environment with the development of cancer is of particular interest and may directly relate to the present study (1,5,8, 13,16). Since particle exposure can inhibit cytotoxic cell function both in vivo (21) and in vitro as noted in the present study, immunosuppression induced by small particles could be of considerable importance.

LGLs, the reputed effector cells responsible of NK and LAK cell activity, are present in many tissues of the body, including the blood, spleen, lymph nodes, lung, and gut (2,3,20,26). Inhibition of LAK and NK cell activity by exposure to particles could decrease the local immune function of those tissues as well as impair the systemic resistance of the host to tumors and infections. Systemic distribution of particles occurs after inhalation, depending on particle diameter (10, 27). This could conceivable result in a more systemic effect of what would be otherwise only local exposure to particulate. The results of the present study suggest that exposure to particles may be a novel mechanism of immunosuppression which may have considerable impact on the ability the host to resist tumors and viral infections.

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