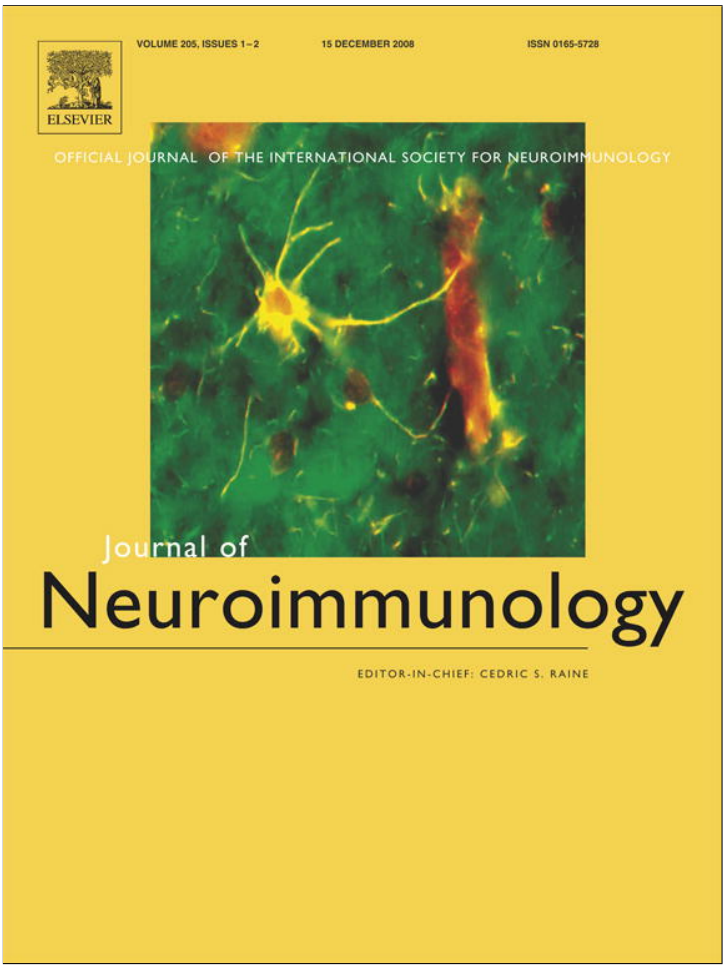

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Low natural killer cell cytotoxic activity in autism: The role of glutathione, IL-2 and IL-15

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ABSTRACT

Although many articles have reported immune abnormalities in autism, NK cell activity has only been examined in one study of 31 patients, of whom 12 were found to have reduced NK activity. The mechanism behind this low NK cell activity was not explored. For this reason, we explored the measurement of NK cell activity in 1027 blood samples from autistic children obtained from ten clinics and compared the results to 113 healthy controls. This counting of NK cells and the measurement of their lytic activity enabled us to express the NK cell activity/100 cells. At the cutoff of 15–50 LU we found that NK cell activity was low in 41–81% of the patients from the different clinics. This NK cell activity below 15 LU was found in only 8% of healthy subjects ($p<0.001$). Low NK cell activity in both groups did not correlate with percentage and absolute number of $CD16^+/CD56^+$ cells. When the NK cytotoxic activity was expressed based on activity/100 $CD16^+/CD56^+$ cells, several patients who had displayed NK cell activity below 15 LU exhibited normal NK cell activity. Overall, after this correction factor, 45% of the children with autism still exhibited low NK cell activity, correlating with the intracellular level of glutathione. Finally, we cultured lymphocytes of patients with low or high NK cell activity/cell with or without glutathione, IL-2 and IL-15. The induction of NK cell activity by IL-2, IL-15 and glutathione was more pronounced in a subgroup with very low NK cell activity. We conclude that that 45% of a subgroup of children with autism suffers from low NK cell activity, and that low intracellular levels of glutathione, IL-2 and IL-15 may be responsible.

1. Introduction

Autism is a developmental disorder of unknown etiology which occurs in childhood (Baird et al., 2003). As with many complex autoimmune diseases, genetic and environmental factors including infection, diet and xenobiotics, along with the resultant immune and neurological abnormalities, are thought to play a role in the development of autism (Edelson and Cantor, 2000; Vojdani et al., 2002; Vojdani et al., 2003; Vojdani et al., 2004; Wiznitzer, 2004; Rutter, 2000; Cohen et al., 2005; Fatemi et al., 2002, 2005; Sebat et al., 2007; Freitag, 2007; Weiss et al., 2008; Kim et al., 2008; Shi et al., 2003). Studies have shown several genetic links, including chromosomal abnormalities, each in a very small percentage of cases (Wiznitzer, 2004; Rutter, 2000; Cohen et al., 2005; Fatemi et al., 2005; Sebat et al., 2007; Freitag, 2007; Weiss et al., 2008; Kim et al., 2008).

Immune abnormalities in autism include changes in the numbers and activities of macrophages, T cells, B cells and natural killer (NK) cell activity (Ashwood et al., 2004; Ashwood et al., 2006; Ashwood and Wakefield, 2006; Stubbs et al., 1977; Warren et al., 1986, 1987). In addition, it has been shown that children with ASD had increased activation of both Th1 and Th2 arms of the adaptive immune response, with a Th2 predominance,

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and without the compensatory increase in the regulatory cytokine IL-10 (Molloy et al., 2006). Another study reported that the innate and adaptive immune responses in children with autism were associated with elevations in TNF-α, IL-1β, and/or IL-6 (Jyonouchi et al., 2001).

In the mid-1970s NK cells were originally identified on a functional basis because of their ability to lyse certain tumor cells in vitro without the requirement for prior immune sensitization of the host (Kiessling et al., 1975; Herberman et al., 1975). Since then, much knowledge has been accumulated regarding their origin, differentiation, receptor repertoire and effector functions, as well as their ability to shape adaptive immune responses (Colucci et al., 2003; Raulet, 2004; Farag and Caligiuri, 2006). NK cells represent a unique subset of lymphocytes, distinct from T and B cells, that contribute to host anti-microbial and anti-tumor defense reactions (Biron et al., 1999; Wu and Lanier, 2001; Mueller et al., 2008). In humans, NK cells are broadly defined as $CD3^{-}CD56^{+}$ lymphocytes. They can be further subdivided into two main subsets on the basis of their surface expression levels of CD56. $CD56^{bright}$ NK cells have predominantly immunoregulatory properties mediated by a potent cytokine producing capacity, while $CD56^{dim}$ NK cells have a marked cytotoxic function (Cooper et al., 2001). The latter cell subset also expresses high levels of the low-affinity Fc receptor for IgG (FcγRIII; also known as CD16) allowing them to mediate antibody-dependent cell-mediated cytotoxicity (ADCC).

NK cells can also be stimulated by several types of cytokines, in particular IL-2 and IL-15. Once activated by cytokines, NK cells proliferate and show increased secretory and cytolytic functions. Activated NK cells secrete interferon-γ (IFNγ), tumor necrosis factor (TNF) and granulocyte/macrophage colony-stimulating factor (GM-CSF). These cytokines, together with secreted chemokines, stimulate inflammatory responses. Cytokine secretion by NK cells also influences adaptive immune responses by modulating the growth and differentiation of monocytes, dendritic cells and granulocytes (Biron et al., 1999; Raulet, 2004). The exocytosis of lytic granules that contain membrane-disrupting proteins such as perforin and a family of structurally related serine proteases known as granzymes is considered to be the main mechanism used by NK cells to kill target cells. NK cells also express molecules of the TNF superfamily that can engage cell-death receptors on target cells, which results in the induction of apoptosis (Bryceson et al., 2006; Hayakawa and Smyth, 2006). Furthermore, there is a correlation between increased NK cell function and anti-tumor responses in individuals treated with IL-2 and IL-15 (Fehniger et al., 2002; Ljunggren and Malmberg, 2007).

In children with autism and the related disorder Rett syndrome, lower levels of circulating numbers of NK cells are noted compared with controls (Raulet, 2004; Farag and Caligiuri, 2006). Additionally, in children with ASD, decreased NK cell activity demonstrated by target lysis has been shown (Warren et al., 1987). This measurement of NK cell activity was carried out on the PBMCs of 31 patients with autism; 12 of the 31 (39%) were found to have reduced levels of cytotoxicity.

Therefore, this multi-center study was conducted on more than one thousand specimens obtained from 10 different clinics in different states. Low NK cell activity was demonstrated in children with autism in all 10 clinics. This low NK cell activity correlated with low intracellular levels of glutathione, a known potentiator of immunological function (Droge et al., 1994), and cytokines such as IL-2 and IL-15 (Ljunggren and Malmberg, 2007).

It seems that a decrease in the cellular cysteine supply decreases the intracellular glutathione (GSH) level. This, along with low levels of IL-2 and IL-15 production, may contribute to lower NK cell activity in autism.

2. Materials and methods

2.1. Study population

Blood samples from 1027 (3:1 ratio of male to female), 2 to 15 years of age, with a diagnosis of autism were sent by different clinicians to our laboratory for immune system evaluation, including NK cytotoxic activity examination. The clinical diagnosis of autism was made according to the DSM IV and/or ICD-10 criteria established by the American Psychiatric Association (Washington, D.C.), as well as by a developmental pediatrician, a pediatric neurologist, and/or a licensed psychologist. Samples were excluded if their medical histories included head injury, evidence of gliomas, failure to thrive, and other known factors that may contribute to abnormal development. Blood samples from 113 children ages 5–15 who came to our laboratory for allergy testing were used as controls. Only individuals with IgE<50 IU and who were completely negative for the tested environmental allergens were used. The study was approved by the Institutional Review Board of the Center for Autism and Related Disorders in Tarzana, CA.

2.2. Preparation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells were prepared from fresh heparinized peripheral venous blood by Ficoll-Hypaque density gradient centrifugation (Sigma-Aldrich, St. Louis, MO). Cells were washed three times with Hank's balanced salt solution (HBSS), and resuspended to 5×10^{6} cells/mL in a complete medium (CM) that consisted of RPMI-1640 supplemented with 10% fetal calf serum and 1% antibiotics (100 U penicillin and 100 μg/mL streptomycin). Purity of the cells was examined by flow cytometry using CD45/CD14 monoclonal antibodies, which was greater than 95%. The viability of cells tested by trypan blue exclusion was greater than 98%. Cells were used for different assays within an hour of isolation.

2.3. Natural killer cell count

NK cell subset enumeration was carried out by using FACScan (Becton Dickinson, Palo Alto, CA). Mononuclear cell populations were determined by two-color direct immunofluorescence and the use of whole-blood staining technique with the appropriate monoclonal antibody and flow cytometry. The fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated monoclonal antibodies (Becton Dickinson) CD16, CD56 PE and CD3-FITC were selected for determination of the total NK cells and NKHT3+/NKHT3− cells. The percentage of positively stained cells for each marker pair, as well as the percentage of doubly stained cells, was determined.

2.4. Natural killer cytotoxic activity by chromium-51 release

A standard 4-h Cr^{51}-release assay was employed (Whiteside et al., 1990). Briefly, 1×10^{4} Cr^{51}-labeled K562 tumor target cells in 0.1 mL complete medium were added to different wells of a microtiter plate. Effector cells were then pipetted into four wells to give effector:target (E:T) ratios of 6:1. 12:1, 24:1 and 48:1, each in triplicate. After a 4-h incubation at 37 °C, the plates were centrifuged at 1400 rpm for 5 min and 0.1 mL of supernatant from each well was collected and placed in a gamma counter. The percentages of isotope released were calculated based on the percentage of tumor cell lysis using the following formula:

$$\%\ \text{Lysis} = \frac{(\text{Experimental Release} - \text{Spontaneous Release})}{(\text{Total Release} - \text{Spontaneous Release})} \times 100$$

Spontaneous release was determined by the addition of the same number of K562 in medium alone. Total releasable radioactivity was determined by incubating an aliquot of Cr^{51} labeled K562 with 1% Triton X-100. Results of NK cell assay for each effector/target ratio can be expressed as a percentage of the specific lysis, but more commonly NK activity is expressed in terms of lytic units (LU). To calculate the LU of NK activity, we used criteria established by Whiteside et al. (1990) for a reproducible NK cell assay. For obtaining the LU of NK activity

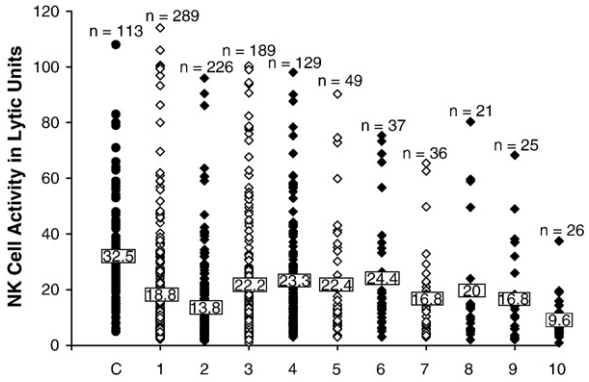

Fig. 1. Distribution of NK cell activity measured in 1027 patients with autism from 10 different clinics (1–10) in comparison to Controls (C). *n*=number of subjects in each group; box point shows mean value.

percentage of specific lysis, all the measured effector target ratios are considered. First, the effector target ratio yielding 20% lysis (E:T 20) is estimated from these measurements. One feature of LU, in contrast with using the percentage of lysis at a single E:T ratio, is that four values at four distinct ratios are used, generating more information and, therefore, greater precision for reproducibility of the assay (Whiteside et al., 1990).

2.5. Intracellular level of glutathione

After Ficoll-Hypaque separation, PBMCs were kept on ice and immediately sonicated for 10 s in 50 mM H_3PO_4 (pH 1.8) that contained 0.1 mM EDTA to minimize auto-oxidation. The pH of the resulting extract was below 2.0. The extract was spun for 5 min at 16,000 g at 4 °C. An equal volume of 200 mM potassium phosphate (pH 9.0) was added to the supernatant followed by treatment with 10 mM 2-Mercaptoethanol at 24 °C. The commercial standard of glutathione (Sigma-Aldrich, St. Louis, MO) was subjected to the same conditions as described above. Both the standards and sample were used to determine intracellular levels by using the HPLC system (ESA Chemsford, MA) Model 5600 coularray detector with solvent delivery pump model 580 and analytical cell that makes use of two porous graphite electrodes. The column was B 3043-13 packed with TSK-gel particles of 5 μ size. The optimal mobile phase was 0.2 M KH_2P0_4/ H_3P0_4 pH 3.0, delivered at 1 mL/min (Rose and Bode, 1995). The detector's response to each concentration of glutathione was determined by making repeated injections of the standards onto the chromatograph. Day to day variability of the quality control specimens was less than 20%. Under these conditions reduced glutathione (GSH) was eluted at 9.5 min and oxidized glutathione (GSSG) at 14.5 min. Glutathione level was expressed by nanogram/3×10^6 PBMCs.

2.6. Effects of glutathione, IL-2 and IL-15 on PBMCs with reduced NK cell activity

Freshly isolated PBMCs from autistic children with NK cell activity below 10 LU were cultured in complete RPMI 1640 and 10% inactivated human serum, with or without IL-2, IL-15 10 ng/mL (R & D Systems, MN) and GSH 20 μg/mL. Cells were incubated for 24 h in CO_2 incubator at 37 °C, then harvested, washed with HBSS, and used for cytotoxic activity by Cr^{51} release assay. NK cell activity of cells incubated in medium alone was compared to cells incubated with IL-2, IL-15 and glutathione.

2.7. Data analysis

Statistics on Software (S.O.S.) version 2 was used of statistical analysis. Normal distribution was tested by the Kolmogorov–Smirnov one-sample test. One-way analysis of variance was performed by means of ANOVA. For post hoc analysis, the large sample *Z*-test was employed. Analysis of population variances was performed using the *F*-test. *p* values were used to determine levels of significance.

3. Results

3.1. NK cell cytotoxic activity in autism

An investigation of the natural killer cytotoxic activity of peripheral blood mononuclear cells from 1027 patients with autism and 113 controls was carried out using Cr^{51} release assay with K562 tumor cells as the target. PMBCs from patients with autism exhibited significantly lower cytotoxic activity than that of the children in the control group. The distribution of the NK cell activity of all groups along with the mean values are presented in Fig. 1. The mean cytotoxic activity for controls was 32.5, and the patients' groups ranged from 9.6 to 23.3 in 9 clinics with *p* values <0.001, and one clinic as high as 24.4 with *p* value 0.01. Analysis of the mean±SD for control specimens (32.5±17.5) and establishment of reference ranges for healthy children (15–50 LU) and its comparison to the autism group allowed us to calculate the percentage of patients with reduced or high NK cell activity. The data presented in Fig. 2 shows that while 8% of healthy controls showed NK cell activity below 15 LU, 41–81% of the autistic group exhibited NK cell activity below the lower reference range ($p<0.001$). This analysis also allowed us to calculate the percentage of individuals with NK cell activity above the higher level of the reference range (50 LU). While 14% of controls had high NK cell activity above 50 LU, 3–14% of patients with a mean of 9% had NK cell activity above this range (calculated from Fig. 1).

3.2. Percentage and absolute number of NK cells for calculation of NK activity per cell

For the determination of NK cell activity per cell percentage and the absolute number of $CD16^+/CD56^+$, cells were determined in 40 blood samples obtained from one out of ten clinics by flow cytometry. The data presented in Table 1 clearly shows that the NK cell number did not always correlate with its lytic activity. For example, patient #8 with an NK cell number of 230 mm^3 has an NK activity of 43.2 LU, and patient #9 has an NK cell number of 418 mm^3, but shows an activity of 13 LU. Likewise, patient #14 with an NK cell number of 177 mm^3 shows an activity of 41.3 LU, while patient #16 with an NK cell number of 1469 mm^3 exhibited the very similar NK lytic activity of 40.4. The conversion of this data into a scatter plot is shown in Fig. 4. The Pearson Correlation Coefficient generated for this figure was (r)=0.246; p=0.126, indicating no relationship between the two variables.

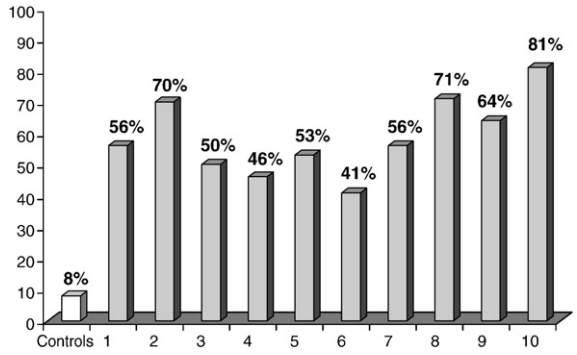

Fig. 2. Percentage of NK cell activity below 15 lytic units in controls and patients with autism obtained from 10 different clinics.

For this reason we expressed the NK cell activity per 100 $CD16^+$/$CD56^+$ cells by using the following formula:

$$\text{NK cell activity/100 } CD16^+/CD56^+ \text{ cells} = \frac{\text{Lytic units of NK cell}}{\text{Absolute number of } CD16^+/CD56^+ \text{ cells}} \times 100$$

Applying this formula to 113 healthy control subjects, we established the NK cell activity per 100 $CD16^+/CD56^+$ cells to be between 5.1 and 10 LU. Analysis of the data in Table 1 indicates that 12 out of 40 samples (42.5%) had lytic units within the normal range of 5.1–10, 5 out of 40 (12.5%) had lytic units >10, and 18 out of 40 (45%) had lytic units <5.1. In the healthy group 78% demonstrated NK cell activity of 5.1–10 lytic units, 14% exhibited lytic units >10, and only 8% had lytic units <5.1. Comparison of 45% of patients with NK lytic units <5.1 with 8% of controls with a similar NK activity resulted in p values <0.0001.

3.3. Correlation between glutathione level and NK cell activity

The relationship between glutathione level and low and high NK cell activity is shown in Fig. 3. The intracellular level of glutathione was measured in PBMCs collected from four different subgroups of patients with NK lytic units of 5–10, 11–20, 21–50 and 51–100. Data from 5 individuals in each subgroup depicted in Fig. 3 shows direct correlation between the cellular level of reduced glutathione and NK lytic activity. For example, PBMCs with NK cell activity of 5–10, 11–20, 21–50 and 51–100 LU showed cellular levels of reduced glutathione at 610±286; 947±458; 1760±895; and 2280±1341 ng/3×10^6 PBMCs respectively. ANOVA was performed for a statistical analysis of the trend, and results showed that the difference between these four groups is significant (F=3.997; p=<0.05).

Table 1
Correlation or lack of correlation between $CD16^+/CD56^+$ cells and NK cytotoxic activity

Specimen number	%$CD16^+$/$CD56^+$	Absolute number $CD16^+/CD56^+$ mm^3	NK cell cytotoxic activity	NK cell activity/100 $CD16^+/CD56^+$ cells
1	5	129	7	5.7
2	10	157	14.7	9.4
3	17	1094	90.2	8.2
4	4	105	13.2	12.6
5	13	355	12.7	3.6
6	21	537	36.5	6.8
7	15	470	20.3	4.3
8	11	230	43.2	18.6
9	19	418	13	3.1
10	10	395	17.6	4.4
11	8	215	14.4	6.7
12	14	636	30.2	4.7
13	12	184	33.9	18.4
14	12	177	41.3	23.3
15	7	126	8.7	6.9
16	28	1469	40.4	2.7
17	15	479	33.9	7.1
18	4	136	7.5	5.6
19	16	511	21.2	4.1
20	7	203	3.6	1.8
21	15	766	74.6	9.7
22	8	513	16.2	3.1
23	28	676	59.9	8.9
24	5	115	5.8	5
25	15	350	33.8	9.6
26	9	320	11.5	3.6
27	5	282	13.3	4.7
28	17	417	30.2	7.2
29	12	323	10.3	3.2
30	11	259	16.8	6.5
31	6	133	8.57	6.44
32	5	150	6.2	4.1
33	12	346	8.9	2.5
34	8	104	20.9	20.1
35	8	168	15	8.9
36	13	375	35.2	9.4
37	18	420	12	2.8
38	8	230	10	4.3
39	26	148	15	9.8
40	6	185	8.6	4.6

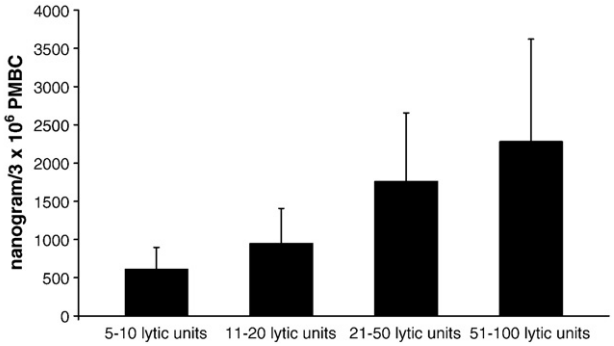

Fig. 3. Correlation between the level of reduced glutathione and low or high natural killer cell cytotoxic activity. ANOVA showed significant differences between the four groups (F=3.997; p=<0.05).

3.4. NK cell culture and treatment with cytokines and glutathione

In investigating NK cell activation after 24-h stimulation with IL-2, IL-15, and glutathione, PBMCs from 12 patients were classified into two groups of 6, with one group having NK cell activity/100 cells of 1.8–5 LU and the other group of 6 with NK cell activity/100 cells of 6–12.6 LU. IL-2, IL-15 and glutathione stimulation of NK cell activity was more pronounced in the group with low NK cell activity/100 cells than in the group with high NK cell activity/100 cells (Table 2).

4. Discussion

Natural killer cells mediate early non-adaptive responses against viruses, intracellular bacteria, parasite-infected cells and malignancies (Deniz et al., 2008; Orange and Ballas, 2006). They mediate these effects through the production of cytokines and the direct killing of a transformed or infected cell by granule release (Kozlowski et al., 1999; Fortier and Kornbluth, 2006; Zhou et al., 2002; Huang et al., 2006).

In addition, these cells are involved in regulating immune response through cytokine production, and altered NK activity is often associated with autoimmune disorders (Johansson et al., 2005). As regulators of adaptive immune response, NK cells inhibit autoreactive T cells to curb neuroinflammation (Segal, 2007; Yamamura et al., 2007).

Although immune system abnormalities have been previously implicated in autism and reported in many articles, natural killer cell activity has only been examined in one study that found reduced activity in 12 of 31 patients. However, this study did not provide evidence for the mechanism responsible for reduced NK cell activity. This reduced killing capacity of NK cells was distributed proportionally between the group of patients with the complete syndrome and those with partial syndrome. Based on this reduction in NK cell activity in about 40% of patients with autism, it was concluded that autism has more than one cause (Warren et al., 1987). Furthermore, they were unable to show a relationship between the level of NK cell activity and NK cell numbers. Although it was hypothesized that low NK cell activity in autism may be due to a deficiency in their interferon production, no attempt was made to explore the mechanism behind this low NK cell activity (Warren et al., 1987).

For this reason, in the present study we investigated the NK cell activity in 1027 blood samples obtained from ten different clinics and different regions of the country and compared the activity to the number of $CD16^+/CD56^+$ cells. Furthermore, we developed a new way

Table 2
The effects of IL-2, IL-15 and glutathione on enhancement of NK cell activity in vitro

Specimen number		Selection based on NK cell activity/100 cells	NK cell activity in medium alone	NK cell activity in medium + IL-2	NK cell activity in medium + IL-15	NK cell activity in medium + glutathione
Subgroup with low NK cell activity	1	5.0	5.8	43	34	27
	2	3.6	12	56	45	30
	3	1.8	4	47	55	63
	4	3.1	13	49	62	35
	5	4.1	6.2	76	53	62
	6	4.3	10	55	40	72
Subgroup with normal NK cell activity	7	6.9	8	26	22	29
	8	12.6	13	16	24	27
	9	8.9	15	23	28	17
	10	9.4	17	20	21	30
	11	6.0	9	7	14	10
	12	9.8	15	33	20	18

to express NK cell activity per cell. Finally, we investigated whether or not IL-2, IL-15 and glutathione play a role in detected low NK cell activity in autism. Since the Warren study measured NK cell activity in only 31 patients generating very low statistical power, we measured the activity of NK cells of blood samples from ten different clinics with the number of patients ranging from 21 to 289 for a total of 1027. While previous testing in our laboratory based on the examination of healthy adults had established the lytic units of NK cell activity to be between 19 and 50, we reexamined this in healthy children and found them to be between 15 and 50 LU. Based on this cutoff we found that NK cell activity could be low in from 41% to as much as 81% of the children diagnosed with autism. This low NK cell activity was not dependent on geographical areas. For example, samples obtained from four clinics in California (Los Angeles, San Diego, San Jose and San Francisco) had low NK cell activity of 41, 71, 50 and 70 respectively, while the low NK cell activity of samples from the east coast ranged from 46 to 56% (Fig. 2). Although in the majority of these cases blood was drawn at the initial appointment before the administration of any form of treatment, these striking differences in NK cell activity from clinic to clinic deserve further investigation as to whether the children received supplementation or any other treatment at home prior to the initial visit.

To find a relationship between low NK cell activity and absolute numbers of $CD16^+/CD56^+$ cells in a subgroup of 40 patients we calculated and expressed the NK cell activity/100 $CD16^+/CD56^+$ cells. Data presented in Table 1 clearly shows that some patients had low absolute numbers of $CD16^+/CD56^+$ cells but had high NK cytotoxic activity, and that some others had high NK cell activity but low absolute numbers. This discordance between absolute numbers of $CD16^+/CD56^+$ cells and NK cell activity is illustrated by a scatter plot, as shown in Fig. 4. Running the Pearson Correlation Coefficient, we obtained $(r)=0.246$; $p=0.126$, indicating no relationship between the two variables.

This calculation enabled us to eliminate the possibility of low or high white blood cell and lymphocyte counts affecting the expression of NK cell activity. Data presented in Table 1 clearly shows that several patients who displayed NK cell activity below 15 LU, after expression of NK cell activity/100 $CD16^+/CD56^+$ cells, exhibited normal NK cell activity of above 5.1. Over all, based on this correction factor and the expression of NK cell activity/$CD16^+$+$CD56^+$ cells, in comparison to 8% of the controls, 45% of children with autism showed low NK cell activity (Table 1).

It has been established that intracellular levels of glutathione can potentiate immunological functions of lymphocytes, including NK cells, in vitro (Droge et al., 1994, 1986; Hamilos and Wedner, 1985; Gmunder and Droge, 1991; Smyth, 1999; Kinscherf et al., 1994). At low GSSG levels, T cells cannot optimally activate the immunologically important transcription factor NF-κB (Pandey et al., 2007), whereas high GSSG levels inhibit the DNA binding activity of NF-κB. NF-κB activation can transduce signals from the T cell antigen receptors, CD4 and CD8 molecules, and from the IL-2 receptor β-chain. The effector phase of cytotoxic T cell responses and IL-2-dependent functions were inhibited even by a partial depletion of the intracellular GSH pool, indicating the importance of the intracellular level of glutathione in NK cytotoxic activity (Liang et al., 1989; Hargrove et al., 1993; Suthanthiran et al., 1990; Galter et al., 1994; Valle Blazquez et al., 1997). Based on these reports, we hypothesized that low NK cell activity may be the result of low intracellular level of glutathione. We examined our hypothesis by measuring the intracellular level of glutathione in a representative sampling of twenty patients, five with low, five with medium, five high and five with very high NK cell activity. The data presented in Fig. 3 shows that low or high cellular levels of glutathione correlate with low or high NK cytotoxic activity. This supports our hypothesis that depletion of glutathione may be one of the factors responsible for the low NK cell activity described in a subgroup of children with autism. Liang et al. in 1989 reported that glutathione regulates the IL-2 activity of cytotoxic T cells. Since then many articles have been published in relation to the potential for IL-2, IL-15 and other cytokines in the regulation of human NK cell survival and function (Carson et al., 1997; Gosselin et al., 1999; Lehmann et al., 2001; Nguyen et al., 2002; Mueller et al., 2008; Hallett et al., 2008).

Indeed, IL-2 and IL-15 are two cytokine and growth factors that regulate lymphocyte function and homeostasis. Early clinical interest in the use of IL-2 in the immunotherapy of renal cell carcinoma and malignant melanoma demonstrated the first efficacy for cytokine therapy in the treatment of neoplastic disease. Advances in our understanding of the cellular and molecular biology of IL-2 and its receptor complex have provided rationale to better utilize IL-2 to expand and activate immune effectors in patients with cancer. Fehniger et al. in 2002 showed that IL-15 uses the IL-2 receptor to activate human NK cells and can synergize with recombinant human IL-12 to stimulate NK cell production of IFN-γ in vitro. IFN-γ production by NK cells is critical in the prevention of overwhelming infection by obligate intracellular microbial pathogens in several experimental animal models (Carson et al., 1997). Based on these findings, we cultured lymphocytes from two subgroups of 6 patients each, one with low NK cell activity/100 $CD16^+/CD56^+$ cells of 1.8–5 LU, and the other group of 6 with normal NK cell activity/100 $CD16^+/CD56^+$ cells of 6.0–12.6 LU, in the presence or absence of 1L-2, 1L-15 and glutathione. Results summarized in Table 2 clearly show that those with lower NK cell activity/cell benefited more from subculture with IL-2, 1L-15 and glutathione. This data further supports the theory that children with autism who demonstrate low NK cell activity/cell may

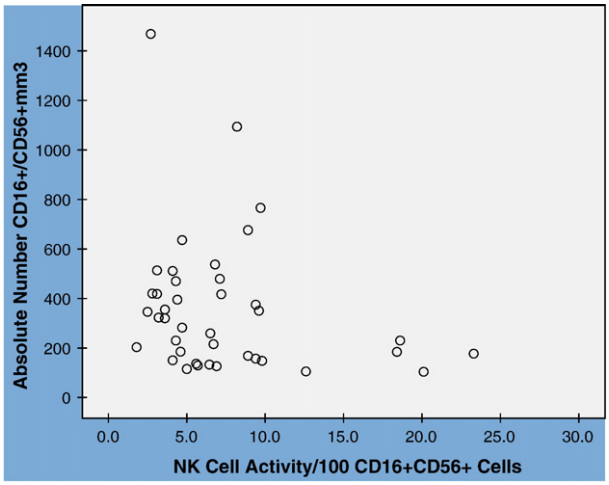

Fig. 4. This scatter plot clearly shows the lack of correlation between the absolute number of $CD16^+/CD56$ mm^3 and NK cell activity/100 $CD16^+/CD56^+$ cells. Pearson Correlation Coefficient $(r)=0.246$; $p=0.126$.

suffer from depletion of glutathione and low intracellular level of IL-2 and IL-15. However, it remains to be proven whether or not supplementation with glutathione and inducers of IL-2 and IL-15 will be able to restore low NK cell activity in children with autism to normal levels. In our earlier studies we had shown that low NK cell activity could be reversed by Vitamin C supplementation in almost 80% of adult patients who suffered from chronic illnesses (Heuser and Vojdani, 1997). Finally, it remains to be investigated whether or not reversal of low NK cell activity can contribute to improved symptomatologies presented in patients with autism. Additional studies are needed to identify the precise mechanism responsible for low NK cell activity in autism, and determine whether its reversal will result in improved functionality in autistic behavior.

Disclosures

Doreen Granpeesheh, Ph.D., B.C.B.A., is the founder and executive director of The Center for Autism and Related Disorders (CARD). She is not on CARD's IRB and took no part in the review process for this study.

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