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NATURAL KILLER CELLS

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SUMMARY

Various factors were reported to influence natural cytotoxicity of natural killer (NK) cells in conventional reared animals. This report summarises the role of specific microbial elements that colonise the alimentary tract of gnotobiotes as a factor in modulation of NK cell activity. Secondly, the effects of diet, age and microbial elements on the NK cell activity of gnotobiotes were examined. Lastly, this study investigated whether microbial enhanced NK cell activity correlated with increased resistance due to systemic infections with the opportunistic fungi and if the systemic fungal infections caused modulation in NK cell activity during infection. Spleen cells from flora defined (FD) and conventionalised (CONV) athymic and euthymic mice show significantly more NK cytotoxicity than germfree (GF), mono-associated (*Candida albicans*, *Streptococcus pneumoniae*, *Streptococcus lactis*, *Morganella morganii*, *Pasteurella pneumotropica*), or di-associated (*Candida albicans* and *Bacillus* sp.) mice. Athymic FD and CONV mice exhibited significantly greater NK cell activity than their euthymic counterparts. Spleen cells from GF mice raised on a chemically defined (low antigen) diet were more cytotoxic towards tumour targets than spleen cells from GF or CV (barrier maintained) mice raised on a natural ingredient diet. No significant differences in NK cell activity were evident in GF or CV mice of different ages. An increased recycling rate of NK cells was observed due to the presence of a microflora. GF and FD mice exhibited significantly elevated NK cell activity after oral association or i.v. challenge with *Cryptococcus neoformans* but not with *C. albicans*. Enhanced NK cell activity did not correlate positively with increased resistance to *Cryptococcus neoformans* infection. Environmental and dietary changes undoubtedly alter the intestinal microflora which can have dramatic affects on the cytotoxic activity of their NK cells.

INTRODUCTION

Lymphocytes that originate from the lymphoid organs or peripheral blood that exhibit "natural" cell-mediated cytotoxicity against tumour cells and a variety of other non-tumour targets are referred to as natural killer (NK) cells.

Natural killer cells are characterised as non-T, non-B large granular lymphocytes that are cytotoxic for a wide variety of target cells including syn-, allo- and xeno-geneic tumour cells, non-malignant cells, virus infected cells and non-tissue targets. Further, NK cells apparently kill without prior exposure, stimuli or manipulation, independent of MHC recognition or immunoglobulin involvement. Because of the implied definition of "natural" killing and non-specific nature of NK cell activation we became interested in whether components of the intestinal microflora directly or indirectly influence the activity and functions of NK cell cytotoxicity.

Various factors were reported to influence natural cytotoxicity of NK cells in conventional reared animals. Factors found to augment NK activity were spontaneous or chemically induced tumours, viruses, bacteria, bacterial components, bacterial adjuvants, interferon and interferon inducers, prostaglandin inhibitors and T-cell growth factors. Factors that suppressed NK activity were certain viruses, carrageenan, cyclosporin A, hydrocortisone, cyclophosphamide, adriamycin, prostaglandins and surgical stress (*Herberman* et al., 1977,1979). Most of these findings were determined in conventional reared animals harbouring an undefined, complex microflora.

This report summarises the role of specific microbial elements that colonise the alimentary tract of gnotobiotes (otherwise devoid of variable, exogenous environmental stimuli), as a factor in modulation of NK cell activity. Secondly, the effects of diet (chemically defined or natural ingredient), age and microbial elements on the NK cell activity of gnotobiotes were examined. Lastly, experimental findings of others have indicated that NK cells may function in natural resistance against certain parasitic (*Kamiyama* et al., 1982), bacterial (*Kasahara* et al., 1981), and viral agents (*Kohl* et al., 1980), as well as NK tumouricidal functions against malignant cells (*Nunn* et al., 1976). Specifically, microbial modulated NK cell activity could play an important role *in vivo*, as an effector mechanism operational against opportunistic infections caused by eukaryotic microorganisms such as the pathogenic fungi *Cryptococcus neoformans* (*Murphy* et al., 1982) and *Candida albicans* (*Marconi* et al., 1985). Natural killer cell activity has correlated positively with the ability of murine NK effector cells to inhibit the growth of fungi *in vitro*. Additional *in vivo* studies by investigators have shown that in conventional (CV) athymic mice (with a complex microbial flora and elevated NK cell activity), the growth of *Cryptococcus neoformans* in tissues was inhibited to a greater extent than in CV euthymic mice (with lower NK cell activity than athymic mice) during the first 7 days following systemic challenge (*Murphy* et al., 1982). Conventional athymic mice were also found to be more resistant to systemic *Candida albicans* infections than euthymic counterparts (*Cutler*, 1976). Cellular components from fungi such as *Cryptococcus neoformans* capsular polysaccharide antigens (*Murphy* et al., 1982) and cell wall constituents from *Candida albicans* (*Carrow* et al., 1985) have been shown to function as immunological modulators, involving both antibody and cell mediated immunity in animals and humans. The objective of these studies were to determine if microbial enhanced NK cell activity correlated with increased resistance due to systemic infections with the opportunistic fungi *Cryptococcus neoformans* or *Candida albicans* and if the systemic fungal infections caused modulation in NK cell activity during the natural course of infections with these agents.

METHODOLOGY

Gnotobiotic animals

Inbred C3H/HeCr, BALB/c (euthymic/athymic), and C57BL/6 (beige/black) mice were used in these studies. Gnotobiotic colonies were established as described previously (*Bartizal* et al., 1983, 1984). Each gnotobiotic mouse colony was housed in a separate isolator and monitored by microbiological methodology. Conventional colonies were maintained in standard animal room quarters. Except for *Cryptococcus neoformans*, all CV and gnotobiotic animals were exposed from birth with their particular microbial flora. Conventionalised (CONV) mice were established by inoculating drinking water of 3-4 week old germfree (GF) mice with 1 ml of a caecal homogenate from a healthy CV mouse. *Cryptococcus neoformans* or *Candida albicans* mice were challenged either orally or intravenously (i.v., tail veins) with 1 LD_{50} log phase, washed yeast cells. All mice were age matched and ranged between 6-10 weeks of age when assayed.

Diets

Mice in dietary studies were divided into 3 groups. In group 1, mice were fed "Amicon thin-channelled", ultra-filtered chemically defined (low-antigen) diet, with a daily lipid supplement. In group 2, mice were fed autoclaved natural ingredient diet. The third group was fed the chemically defined diet for approximately 11-15 weeks and then switched to autoclaved natural ingredient diet for the remainder of the study.

Natural cytotoxicity assays

Effector and target cell suspensions were prepared as described previously (*Bartizal* et al., 1983). Natural killer cell activity was determined by a direct 4 h ^{51}Cr-release assay at various effector:target ratios. Lymphocytes derived from spleens of animals in different experimental groups were performed in quadruplicate. Supernatant samples were collected using the Titertek supernatant collection system (Flow Laboratories) and counted in an autogamma spectrometer. Percent cytotoxicity was calculated as follows:

$$\%\text{cytotoxity} = \frac{\text{experimental c.p.m. - spontaneous c.p.m.}}{\text{maximum c.p.m. - spontaneous c.p.m}} \times 100$$

Significance of data was analysed (Student's t-test and analysis of variance) from quadruple experimental samples, from at least 3 age-matched mice from an experimental group, assayed on the same day.

In addition, a modified single cell NK assay was used to determine NK cell parameters. Briefly, splenic NK lymphocytes and YAC-1 tumour target cells were used at 1 effector:2 target cell ratio. Effector and target cells were incubated together to form conjugates, pelleted and mixed with molten agarose then spread over agarose pre-coated slides and incubated for 4 h. Results were assessed microscopically using trypan blue dye exclusion to determine cell viability. The NK cell parameters determined were mean percent target binding cells (%TBC), mean percent target binding cells that were cytotoxic (%TBCC), and mean percent active NK cell frequency (%NK). Calculations used to derive parameters were described previously (*Liljequist* et al., 1987).

Table 1: Cytotoxicity in BALB/c mice by the ^{51}Cr-release assay

Microbial status	Phenotype	Percent Cytotoxicity[a]
Germfree	athymic	14.25[b]
	euthymic	9.76[b]
C. albicans MA	athymic	12.30[b]
	euthymic	10.80[b]
S. pneumoniae MA	euthymic	3.74[b]
S. lactis MA	euthymic	4.47[b]
M. morganii MA	euthymic	4.88[b]
P. pneumotropica MA	euthymic	4.37[b]
C. albicans + *Bacillus* sp. DA	athymic	12.54[b]
	euthymic	10.16[b]
Flora defined	athymic	31.83
	euthymic	21.21
Conventionalised	athymic	43.85
	euthymic	24.92

[a]: effector:target ratio = 100:1.
[b]: Significantly lower ($p<0.05$) than flora defined and conventionalised (mean values).

RESULTS AND DISCUSSION

Previous studies of NK cell activity in mice have not resolved whether differences exist in GF, gnotobiotic or CV mouse NK cell cytotoxicity. These studies demonstrate that spleen cells from flora defined (FD) and conventionalised (CONV) athymic and euthymic mice show significantly more NK cytotoxicity than germfree (GF), mono-associated (*Candida albicans*), or di-associated (*Candida albicans* and *Bacillus* sp.) mice (see Table 1). Spleen cells from euthymic GF mice had significantly less NK cell activity compared to athymic GF mice. Athymic FD and CONV mice exhibited significantly greater NK cell activity than their euthymic counterparts. Colonisation of the alimentary tract of GF athymic or euthymic mice with pure cultures of *Candida albicans*, *Streptococcus pneumoniae*, *Streptococcus lactis*, *Morganella morganii*, *Pasteurella pneumotropica*, alone or *Candida albicans* in combination with a *Bacillus* sp., did not significantly alter the NK cytotoxicity of their splenic lymphocytes even though these microbes were present in high numbers (10^{8-10}/g) within their alimentary tracts. Percent cytotoxicity also increased as the effector:target ratio increased. These studies indicate that the intestinal microflora can alter murine natural NK cell cytotoxicity (Table 1).

Data in Table 2 suggest that spleen cells from GF mice raised on a chemically defined (low antigen) diet were more cytotoxic towards tumour targets than spleen cells from GF or CV (barrier maintained) mice raised on a natural ingredient diet. The NK cell activity of GF mice was dramatically increased after alimentary tract colonisation with a complex CV intestinal microflora. Conventional mice raised in clean (barrier) conditions showed significantly less NK cell activity than non barrier-reared mice. Switching GF mice from a chemically defined diet to a natural ingredient diet did not enhance NK cell

Table 2: The effects of age, diet and microbial flora on NK cell activity in C3H/HeCr mice

Microbial status	Diet[a]	Age (wks)	Percent cytotoxicity[b]
Germfree	CD	6-7	16.63
Conventional	Nl	7	9.33
Germfree	Nl	9-10	8.22
Germfree	CD-NI	15-19	11.18
Germfree	CD	29-35	16.28
Germfree	Nl	30-32	11.06
Conventional[c]	NI	34-36	9.52
Conventionalised	Nl	7-8	19.88[d]
	CD-NI	8	51.26 [d]

[a]: CD= chemically defined, NI = natural ingredient, CD-NI = diet switch.
[b]: effector:target ratio = 100:1.
[c]: CV are barrier reared (clean conventional).

activity. No significant differences in NK cell activity were evident in GF or CV mice of different ages (6-10 weeks old vs. 29-36 weeks old). These results indicate that the diet and microbial flora modulate the NK cell activity of mice. Most mice purchased from commercial suppliers are designated as being "flora defined" or "specific pathogen free". After equilibration into new animal quarters these mice are usually fed different diets and acquire different species of environmental microbes. These environmental and dietary changes undoubtedly alter the intestinal microflora, which can have dramatic affects on the cytotoxic activity of their NK cells.

When NK cell parameters were evaluated in the single cell assay, no significant differences in target binding ability (%TBC) were found between GF or CV mouse spleen cells for either athymic or euthymic mice (Table 3). However, the %TBC of athymic mouse cells was significantly greater than euthymic mouse cells. Significantly lower cytotoxicity (%TBCC) was found in non-NK-enriched populations of GF and CV athymic or euthymic spleen cells. Only the spleen cells from NK-enriched GF euthymic mice showed significantly lower %TBCC compared to CV euthymic and GF or CV athymic cells from the same population. The frequency of active NK cells (%NK) appeared higher in both GF and CV athymic mice compared to GF or CV euthymic mice in either enriched or non-enriched NK spleen cell populations. Enriched populations of athymic CV effector cells showed significantly greater %NK than non-enriched athymic CV effector cells. These results emphasise that binding ability (%TBC) is influenced by mouse genotype (athymic or euthymic) and is augmented in the presence of a microflora.

The single cell cytotoxicity assay prevents NK cells from recycling in agarose medium. The observed augmentation of NK cell activity due to microbial influence may represent an increased recycling rate of CV effector NK cells which are detected in the single cell assay. The increased %NK (calculated as a factor of %TBC and %TBCC) of GF and CV athymic and euthymic mice is largely a result of the increased %TBC factor, since %TBCC is relatively equal as measured for athymic and euthymic mice. Therefore, an increased recycling rate of NK cells

Table 3: Cytotoxicity in BALB/c mice determined by the single cell assay

	Microbial Status	Phenotype	%TBC	%TBCC	%NK
Nylon wool enriched:					
	CV	athymic	20.6	17.3	3.6
	GF	athymic	18.4	17.8	3.1
	CV	euthymic	11.7	17.3	2.0
	GF	euthymic	11.0	12.4	1.4
Non enriched:					
	CV	athymic	27.9	8.9	2.4
	GF	athymic	29.5	9.6	2.7
	CV	euthymic	18.6	8.1	1.5
	GF	euthymic	18.5	8.8	1.6

may occur due to the presence of a CV microflora. Suppressor cells present in euthymic spleen cell populations may down-regulate %TBC. Since NK cells are unable to recycle in the single cell assay (in agarose) the effects of down-regulation may become more apparent in the single cell assay compared to the chromium release assay.

Both GF and FD athymic and euthymic mice exhibited significantly elevated NK cell activity after oral association or i.v. challenge with *Cryptococcus neoformans* (Table 4). Augmented NK cell activity occurred rapidly (24 h) after challenge and persisted for 14 days post challenge. Live and heat-killed *Cryptococcus neoformans* or its capsular polysaccharide enhanced NK cell activity in mice. *Cryptococcus* augmented NK cell activity was seen to a greater extent in GF athymic mice than in euthymic mice, while in FD mice the opposite effect was seen. The greatest augmentation of NK activity occurred in systemically (i.v.) challenged mice administered viable *Cryptococcus neoformans* cells, with lesser augmentation seen in orally associated mice. The least NK cell augmentation was observed in capsular polysaccharide injected mice. Augmented NK cell activity did not appear to correlate with immunity to cryptococcal infection since all mice eventually succumbed to disseminated cryptococcal infection. The route of

Table 4: Augmentation of splenic NK cell activity after *C. neoformans* challenge in BALB/c mice (^{51}Cr-release assay).

	Challenge	% Cytotoxicity at E:T Ratio of 100:1					
		Day 3		Day 7		Day 14	
		Ath	Euth	Ath	Euth	Ath	Euth
Germfree:	capsule i.v.	6.9	4.2	11.0	2.8	7.9	0.0
	live oral	12.5	8.6	16.3	5.9	34.6	5.4
	live i.v.	26.7	16.3	39.6	23.2	37.1	6.6
Flora-defined:	live i.v.	1.5	0	15.8	28.8	all died	41.6
	heat-killed i.v.	9.0	13.1	4.2	6.3	n.d.	n.d.

Table 5: NK cell activity and lethality after systemic i.v. challenge of gnotobiotes with *C. albicans* or *C. neoformans*

Microbial status	Strain/phenotype	% Cytotoxicity at E:T Ratio 100:1 Before challenge	7-21 days after challenge[a]	Survival after challenge[b]
C. neoformans challenged mice:				
Germfree	BALB/Euthymic	7.04	13.44[c]	38
Germfree	BALB/Athymic	12.35	42.52[c]	13
Flora defined	BALB/Euthymic	18.67	30.91[c]	15
Flora defined	BALB/Athymic	16.78	29.67[c]	12
Germfree	C57BL/6/black	9.95	2.49[d]	19
Germfree	C57BL/6/beige	1.09	2.90	17
Conventional	C57BL/6/black	16.73	23.05	19
Conventional	C57BL/6/beige	5.69	4.35	17
C. albicans-challenged mice:				
C. albicans MA	BALB/euthymic	10.73	6.59	no dead
C. albicans MA	BALB/athymic	16.23	16.81	no dead

[a]: Cytotoxicity was determined 7-21 days post challenge on survivors.
[b]: Mortality represents data from 12-24 mice per group from a 50 day period.
[c]: Significantly greater ($p<0.01$) than unchallenged mice of same genotype and assay time.
[d]: Significantly less ($p<0.05$) than unchallenged mice of same genotype and assay time.

challenge, host immunocompetence, nature of the antigen and host microbial status were found to be important factors in NK cell modulation by the opportunistic fungi. Additionally, enhanced NK cell activity did not correlate positively with increased resistance to *Cryptococcus neoformans* infection. In contrast, athymic mice challenged i.v. with *Candida albicans* showed enhanced NK cell activity compared to non challenged, *Candida albicans* mono-associated mice. Euthymic mice, however, showed unaltered NK cell activity following i.v. challenge (Table 5). No mortality was observed in either athymic or euthymic mice challenged i.v. with *Candida albicans* during the experimental period.

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