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Flow cytometric and morphologic studies of HNK1$^+$ (Leu 7$^+$) lymphocytes in relation to cytomegalovirus carrier status

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SUMMARY

CMV carrier status of healthy individuals causes a persistent increase in the numbers of lymphocytes expressing the HNK1 (Leu 7) antigen. We investigated this phenomenon by correlating HNK1 fluorescence intensity of the CD3$^+$, CD4$^+$, CD8$^+$ and CD16$^+$ lymphocytes with their morphology. The study was performed on 112 healthy individuals as a function of their CMV carrier status. To this end, the CD3$^+$, CD4$^+$, CD8$^+$ and CD16$^+$ lymphocytes were stratified into subsets with undetectable (−), weak (+) and strong (++) levels of HNK1 fluorescence intensity, as well as into subsets with low, intermediate or high perpendicular light scatter (PLS) signals. Among the CD3$^+$, CD4$^+$ and CD8$^+$ lymphocytes. CMV carrier status was associated with significantly increased numbers of HNK1(+) and particularly HNK1(++) cells. Among the CD16$^+$ lymphocytes, CMV carrier status increased only the HNK1(++) ones significantly. There were clear positive correlations between the PLS characteristics of the CD3$^+$, CD4$^+$, CD8$^+$ and CD16$^+$ lymphocytes and their HNK1 fluorescence intensity. Microscopic analysis of CD3$^+$ lymphocytes sorted into HNK1(−), HNK1(+) and HNK1(++) fractions revealed that the percentage of those cells containing azurophilic granules and the numbers of such granules per cell increased in proportion to their HNK1 fluorescence intensity. Thus, CMV also interacts with the HNK1$^+$ lymphocytes of its healthy carriers by increasing the level of expression of that antigen which is accompanied by increased granularity. Those granular lymphocytes may include cytotoxic cells which are involved in the control of viral replication.

Keywords cytomegalovirus lymphocyte subpopulations monoclonal antibodies flow cytometry morphology

INTRODUCTION

An immunologic consequence of active cytomegalovirus (CMV) infection in recipients of allogeneic heart and bone marrow transplants is an increase in CD8$^+$ T lymphocytes which express the HNK1 (Leu 7) antigen (Maher *et al.*, 1985; Würsch *et al.*, 1985). The high numbers of CD8$^+$ HNK1$^+$ T cells may persist for years in those patients suggesting a continuous interaction between CMV and their immune system. A comparison of the T cell phenotypes of CMV seropositive and CMV seronegative healthy individuals revealed a similar influence of CMV carrier status, but of a much smaller magnitude than in the transplant recipients (Gratama *et al.*, 1987a; 1987b). CMV seems to be unique among the herpes viruses in that respect, since the carrier status of Epstein–Barr virus, herpes simplex virus or varicella-zoster virus is not associated with increased numbers of HNK1$^+$ T cells (Gratama *et al.*, 1987b).

Subsets of HNK1$^+$ lymphocytes have been distinguished on the basis of phenotyptic, morphologic and functional criteria (Lanier *et al.*, 1983; Abo, Miller & Balch, 1984). One subset consists of CD3$^-$, CD16$^+$ lymphocytes which have abundant azurophilic granules in their cytoplasm and a high level of natural killer (NK) activity against K562 erythroleukemia cells. The other subset consists of CD3$^+$, CD16$^-$ lymphocytes with few cytoplasmic granules and a low level of NK(K562) activity. Our previous study revealed that CMV carriers have larger numbers of HNK1$^+$, CD16$^-$ lymphocytes than non-carriers, but that the numbers of HNK1$^+$, CD16$^+$ lymphocytes and NK(K562) activity are essentially similar (Gratama *et al.*, 1987a).

We report here the results of our study on the influence of

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CMV carrier status on HNK1 fluorescence intensity of peripheral blood lymphocyte subsets in relation to their flow cytometric and morphologic characteristics.

MATERIALS AND METHODS

Healthy volunteer blood donors

One-hundred and twelve healthy individuals (58 males and 54 females) participated in the study. Their ages ranged from 18 to 63 years: 68 were blood donors of the Dutch Red Cross Blood Transfusion Service and 44 were hospital personnel.

Assessment of CMV carrier status

We took the presence of IgG-class antibodies against CMV late antigen in the sera of the blood donors as a marker for their CMV carrier status. Those antibodies were detected using an indirect enzyme-linked immunosorbent assay (ELISA) (Middeldorp et al., 1984). IgM-class antibodies against CMV late antigen were determined using a direct ELISA (Sundqvist & Wahren, 1981; Sundqvist, 1982).

Flow cytometric studies

Mononuclear cells were isolated from heparinized venous blood samples using Ficoll-Isopaque density gradient centrifugation. These samples were drawn between 8 and 11 am in order to exclude the influence of circadian variations on lymphocyte subsets (Ritchie *et al.*, 1983).

For the study of the co-expression of HNK1 by the $CD3^+$, $CD4^+$, $CD8^+$ and $CD16^+$ lymphocytes, mononuclear cells were incubated with mixtures of biotinylated Leu 7 (an IgM HNK1 monoclonal antibody (MoAb)) and one of the following MoAb: Leu 4 (an IgG1 CD3 MoAb), Leu 3a (an IgG1 CD4 MoAb), FK18 (an IgG3 CD8 MoAb) and Leu 11a (an IgG1 CD16 MoAb). The Leu 11a MoAb had already been conjugated with fluorescein isothiocyanate (FITC). After washing once, the cells were incubated with the appropriate mixtures of streptavidin conjugated with phycoerythrin (PE) and subclass-specific goat-anti-mouse antibodies conjugated with FITC for 30 min at 0°C. All MoAb and conjugates were used at saturating concentrations. The Leu MoAb and the streptavidin were purchased from Becton Dickinson (Mountain View, CA). FK18 was purchased from the National Institute of Public Health and Environmental Hygiene (Bilthoven, the Netherlands) and all goat-anti-mouse antibodies were purchased from Nordic Immunological Laboratories (Tilburg, the Netherlands).

After the final washing step, flow cytometry was performed on a FACS IV fluorescence-activated cell sorter (Becton Dickinson) equipped with an argon-ion laser tuned at 488 nm with an intensity of 300 mW. Fluorescence signals for each fluorochrome were detected selectively using a 530 nm band pass filter for green (FITC) emission and a 575 nm band pass filter for orange-red (PE) emission. If necessary, correction for the spectral overlap of those two wavelengths was performed by using the dual compensation network of the FACS IV. The peak amplitudes of the signals from the forward angle light scatter (FLS), perpendicular light scatter (PLS), green fluorescence and orange-red fluorescence were converted from analog to digital, and were amplified linearly (FLS and PLS) or logarithmically over 1·5 decades (green and orange-red fluorescence). Those digital signals were split into 256 channels by a pulse height analyser. Living lymphocytes were electronically selected on the basis of their FLS and PLS characteristics.

All flow cytometric studies were performed after careful alignment of the FACS IV according to a standardized set-up procedure using fluorescent calibration beads (Polysciences, Warrington, PA, USA). That procedure allowed an appropriate comparison of data obtained at different days.

Data on FLS, PLS, green and orange-red fluorescence signals of 25,000 lymphocytes per sample were stored in list

Table 1. Correlation between HNK1 fluorescence intensity and CMV carrier status

Lymphocyte subset	CMV seropositive* ($n=53$)	CMV seronegative* ($n=59$)	*P* value
$CD3^+$,HNK1(−)	1341 (749, 2275)	1224 (723, 2175)	NS
$CD3^+$,HNK1(+)	89 (24, 376)	47 (13, 133)	0·0001
$CD3^+$,HNK1(++)	58 (10, 379)	20 (<5, 123)	0·0001
$CD4^+$,HNK1(−)	996 (561, 2071)	908 (513, 1606)	NS
$CD4^+$,HNK1(+)	27 (6, 211)	17 (<5, 97)	0·01
$CD4^+$,HNK1(++)	25 (<5, 105)	9 (<5, 38)	0·0001
$CD8^+$,HNK1(−)	527 (249, 1129)	438 (211, 964)	NS
$CD8^+$,HNK1(+)	63 (16, 296)	38 (14, 142)	0·03
$CD8^+$,HNK1(++)	49 (10, 296)	16 (<5, 132)	0·0001
$CD16^+$,HNK1(−)	193 (61, 483)	234 (118, 549)	NS
$CD16^+$,HNK1(+)	73 (14, 265)	67 (18, 210)	NS
$CD16^+$,HNK1(++)	31 (<5, 187)	16 (<5, 88)	0·004

* Results expressed as median (5th and 95th percentiles) number of lymphocytes/mm^3.

P values were determined using standard regression analyses that controlled for age, gender and CMV carrier status (see Materials & Methods).

NS, not significant.

mode for analysis using a Consort 30 system (Becton Dickinson). In the first step of those analyses living lymphocytes were selected by gating on the FLS and PLS parameters. Correlated data of green and orange-red fluorescence were then collected into 64 × 64 matrices and displayed as contour plots. Those plots were divided into quadrants representing unstained cells, cells stained with both fluorochromes or cells stained with either PE- or FITC-labelled MoAb. The PE-positive quadrants were further subdivided into HNK1(+), i.e., 1/3 with relatively dim fluorescence, and HNK1(++), i.e., the remaining 2/3 with bright fluorescence. With the amplification over 1·5 log decades, that subdivision corresponded to cell populations with relative fluorescence intensities ranging from 1·5 to 4·1 arbitrary units and from 4·3 to 31·6 arbitrary units respectively. The absolute numbers of the different lymphocyte subpopulations were calculated from the percentages of lymphocyte subsets obtained by flow cytometry and from the simultaneously obtained absolute lymphocyte counts, which were determined using a Hemalog D cell counter (Technicon, Tarrytown, NY, USA).

In the second step gates were set on the FLS and FITC parameters and the $CD3^+$, $CD4^+$, $CD8^+$ and $CD16^+$ lymphocytes were divided into those with low, intermediate and high PLS signals. To this end, the linear PLS scale was divided into three equal parts. The HNK1 fluorescence intensity of each of the resulting 12 lymphocyte subsets was then studied after generation of histograms of PE fluorescence. The intensity of HNK1 immunofluorescence of the HNK1 positive cells was analysed by computing the weighted mean channel of HNK1 immunofluorescence for each histogram.

Morphologic studies

Mononuclear cells were incubated with a mixture of Leu 7/FITC and Leu 4/PE and fractionated into $CD3^+$,HNK1(−), $CD3^+$,HNK1(+) and $CD3^+$,HNK1(++) subsets. During the sorting procedure the cell rate was kept below 2,500 cells/s in order to obtain optimal purity of the sorted cell fractions. The purity of those fractions was assessed microscopically and was generally >95%. From the sorted fractions, cytocentrifuge preparations were made, stained with May–Grünwald–Giemsa and examined microscopically. At least 300 cells per slide were studied.

Statistical analyses

Inspection of the distribution curves of the lymphocyte subset counts and related variables revealed that many of them were non-Gaussian. Therefore, non-parametric statistics were used for the univariate analyses of the effects of age, gender and CMV carrier status on those parameters. In accordance with previous reports (Abo, Cooper & Balch, 1982; Ligthart *et al.*, 1986; Gratama *et al.*, 1987a; 1987b), age and gender influenced significantly some of the lymphocyte subset counts in the present study. Therefore, the effects of CMV carrier status on lymphocyte subset counts were further studied using standard linear regression procedures that controlled for age, gender and CMV carrier status. Before these analyses the lymphocyte subset data were transformed to their natural logarithms in order to reduce skewness. The results of the PLS studies were analysed using the signed rank test. Only P values $<0·05$ were considered significant.

Table 2. Correlation between HNK1 fluorescence intensity and perpendicular light scatter signal

Lymphocyte subset	Perpendicular light scatter signal*		
	Low	Intermediate	High
$CD3^+$	83 (46, 151)	100 (48, 167)	146 (54, 194)
$CD4^+$	88 (56, 142)	100 (40, 168)	141 (50, 191)
$CD8^+$	89 (54, 158)	106 (56, 176)	150 (61, 193)
$CD16^+$	75 (46, 127)	101 (49, 164)	144 (55, 191)

* Results expressed as median (5th and 95th percentiles) channel of HNK1 fluorescence of HNK1 positive cells in 112 individuals. For computation, see Materials & Methods. The differences between HNK1 positive lymphocytes with low and intermediate PLS signals, and between those with intermediate and high PLS signals were significant for all four subsets ($P<0·001$ in all cases with the signed rank test).

RESULTS

Correlation between CMV carrier status and HNK1 (Leu 7) fluorescence intensity

Fifty-three of the 112 individuals had IgG-class CMV antibodies and none had IgM-class CMV antibodies. The 53 CMV seropositive individuals had higher numbers of $CD3^+$, $CD4^+$, $CD8^+$ and $CD16^+$ lymphocytes expressing HNK1 in their peripheral blood than the 59 CMV-seronegative individuals (Table 1). Inspection of HNK1 fluorescence intensity revealed that the effects of CMV carrier status were most significant on the small proportions of $CD3^+$, $CD4^+$, $CD8^+$ and $CD16^+$ lymphocytes with strong HNK1 fluorescence (HNK1(++)). The effects of CMV carrier status on the somewhat larger subsets of $CD3^+$, $CD4^+$ and $CD8^+$ lymphocytes with dim HNK1 fluorescence (HNK1(+)) were generally less significant, and the numbers of $CD16^+$, HNK1(+) lymphocytes were similar in both groups. The effects of CMV carrier status on the majority of the $CD3^+$, $CD4^+$, $CD8^+$ and $CD16^+$ lymphocytes, i.e., those with undetectable HNK1 fluorescence (HNK1(−)), were not significant.

Correlation between PLS characteristics and HNK1 fluorescence intensity

In order to study the correlation between HNK1 fluorescence intensity and cell morphology we divided the $CD3^+$, $CD4^+$, $CD8^+$ and $CD16^+$ lymphocytes into subsets with low, intermediate and high PLS signals. The PLS signal is mainly determined by the internal cell structure (Salzman, Growell & Martin, 1975). Most of these lymphocytes had low PLS signals. The correlations between the PLS characteristics of the HNK1 positive (HNK1(+) and HNK1(++)) lymphocytes among the $CD3^+$, $CD4^+$, $CD8^+$ and $CD16^+$ subsets and their HNK1 fluorescence intensities are shown in Table 2. To analyse these correlations we computed the weighted mean channel of PE (HNK1) fluorescence of the $HNK1^+$ lymphocytes and used this parameter as an indicator of the level of HNK1 expression by these cells. Among all four subsets ($CD3^+$, $CD4^+$, $CD8^+$ and $CD16^+$), there were similar and clear positive correlations between PLS signals and HNK1 fluorescence intensities. The differences in HNK1 fluorescence intensity between the lympho-

cytes with low and intermediate PLS signals, and between those with intermediate and high PLS signals were significant in all four lymphocyte subsets ($P < 0{\cdot}001$ in all cases with the signed rank test). The correlations between PLS signals and HNK1 fluorescence intensities were similar in CMV-seropositive and CMV-seronegative individuals (data not shown).

Correlation between HNK1 fluorescence intensity and morphology of CD3$^+$ lymphocytes

The positive correlation between HNK1 fluorescence intensity and PLS signal prompted us to study the morphology of CD3$^+$ lymphocytes in relation to their HNK1 fluorescence intensity. We selected the CD3$^+$ lymphocytes for our sorting experiments for two reasons. Firstly the proportion of that subset was sufficient to allow sorting into HNK1(−), HNK1(+) and HNK1(++) fractions in most cases, and secondly the influence of CMV carrier status on HNK1 expression was most evident for the CD3$^+$ lymphocytes. The results from five CMV-seronegative and five CMV-seropositive individuals are set out in Table 3. In seven individuals (five CMV-seropositive and two CMV-seronegative) the fractions of CD3$^+$ lymphocytes with azurophilic granules increased proportionally to their HNK1 fluorescence intensity.

The morphology of the CD3$^+$ lymphocytes in relation to their HNK1 fluorescence intensity is illustrated in Fig. 1. The CD3$^+$,HNK1(−) subset consisted of small lymphocytes characterized by mainly round, clefted nuclei, coarse chromatin and very small amounts of cytoplasm with almost no azurophilic granulation. The CD3$^+$,HNK1(+) lymphocytes were somewhat larger with round or irregular nuclei, moderately condensed chromatin and a medium amount of pale-blue cytoplasm containing fewer than 10 azurophilic granules per cell in 50 to 70% of the cells. The CD3$^+$,HNK1(++) subset consisted of medium-sized lymphocytes with similar nuclear morphology as the CD3$^+$HNK1(+) lymphocytes and large amounts of pale-blue cytoplasm. The majority of the CD3$^+$,HNK(1++) lymphocytes showed many cytoplasmic granules (5 to 20 per cell).

DISCUSSION

CMV-seropositive individuals have significantly higher numbers of HNK1 (Leu 7) positive lymphocytes than CMV-seronegative individuals. This effect of CMV carrier status involves lymphocytes expressing T cell markers (CD3, CD4, CD8) and showing dim HNK1 fluorescence, but it is most evident for those lymphocytes with strong HNK1 fluorescence, including the CD16$^+$ ones. Lymphocyte morphology correlates with HNK1 fluorescence intensity. Flow cytometric studies of CD3$^+$, CD4$^+$, CD8$^+$ and CD16$^+$ lymphocytes reveal an increase in their PLS signals, and microscopic studies of CD3$^+$ lymphocytes show an increase in their granularity proportional to their HNK1 fluorescence intensity. Our results confirm those of Terstappen *et al.* (1986) who reported that among the CD8$^+$ lymphocytes, the HNK1 positive ones had higher PLS signals than the HNK1 negative ones.

The combined results of our flow cytometric and morphologic studies indicate that the shape of the nucleus and, particularly, the presence of increasing numbers of cytoplasmic granules (detected after May–Grünwald–Giemsa staining) contribute to the increasing PLS signal as a function of HNK1 expression. In addition, cytoplasmic granules that are undetectable after May–Grünwald–Giemsa staining may also have increased the PLS signal. There is no absolute correlation between cytoplasmic azurophilic granules and HNK1 expression. This conclusion can be drawn from the description of CD16$^+$,HNK1$^-$ granular lymphocytes in healthy individuals (Abo, Miller & Balch, 1984), agranular CD8$^+$,HNK1$^+$ T cells in allogeneic marrow graft recipients (Leroy *et al.*, 1986) and the presence of small proportions of granular cells among our CD3$^+$,HNK1(−) lymphocytes (Table 3). Abo, Cooper & Balch (1982) described cytoplasmic HNK1 expression in some but not all cells that expressed HNK1 on their surface. Thus, the presence of cytoplasmic azurophilic granules and the expression of HNK1 on the cell surface appear to be closely related but independent phenomena.

The presence of cytoplasmic granules is correlated with the lytic capability of those cells (Henkart, 1985) and suggests, in this context, a role for HNK1$^+$ lymphocytes in combatting CMV infection. HNK1 itself is an epitope on the carbohydrate moiety of several neural cell adhesion molecules and is involved in the interactions between those cells (Künemund *et al.*, 1988). Similarly, the HNK1 epitopes may serve as interaction sites between cytotoxic lymphocytes and their targets. Within that perspective, the close correlation between cytoplasmatic granularity and membrane HNK1 expression would be of interest.

Borysiewicz *et al.* (1983, 1988) have shown that class I MHC-restricted, CMV-specific cytotoxic T cell precursors express the CD8 marker and occur at a frequency of 1 per 5,000 to 20,000 peripheral T lymphocytes. Whether or not these cells express HNK1 is unknown. MHC-unrestricted killing of CMV-infected fibroblasts is mediated by CD16$^+$,HNK1$^+$ lymphocytes (Borysiewicz *et al.*, 1985). In this context it is interesting to

Table 3. Correlation between HNK1 fluorescence intensity and morphology of CD3$^+$ lymphocytes (% of lymphocytes with azurophilic granules)

	CMV-seropositive donors					CMV-seronegative donors				
CD3$^+$ lymphocyte subset	F,52	F,32	M,42	M,29	M,35	F,32	F,24	M,33	M,44	F,37
HNK1(−)	3	4	6	9	3	1	7	9	11	4
HNK1(+)	55	49	52	54	49	72	78	51	47	49
HNK1(++)	79	78	81	67	77	NE	70	78	53	NE

Each donor is indicated by gender (M or F) and age (years).
NE, not evaluable (subset contained too few cells to enable sorting).

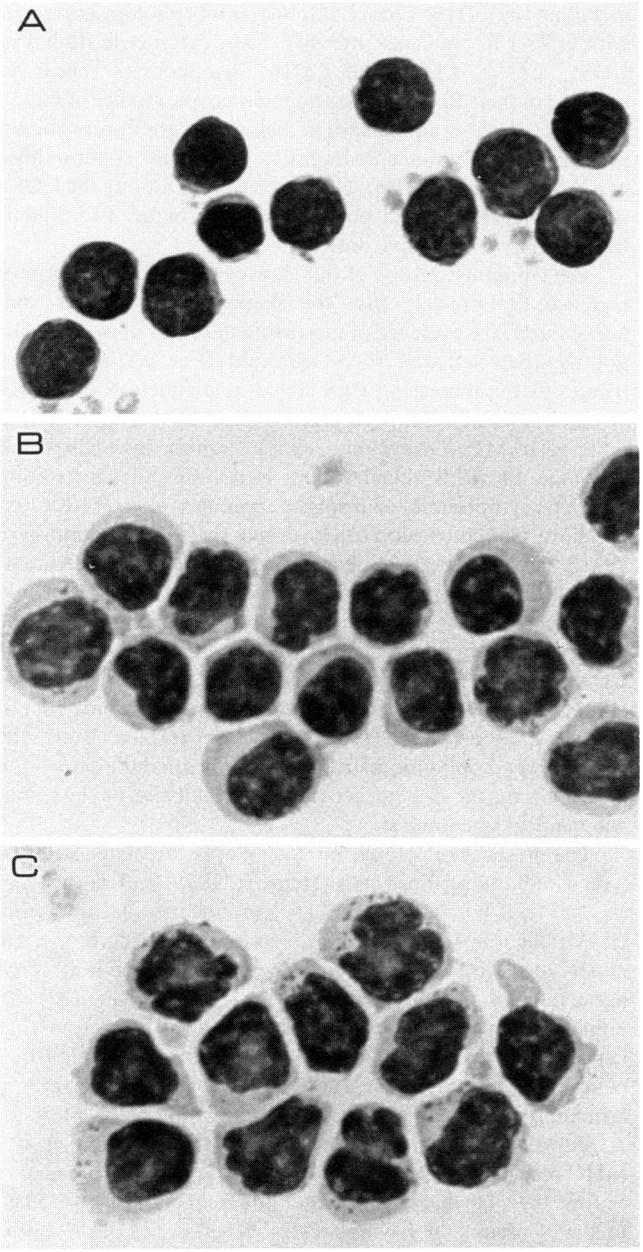

Fig. 1. Cytocentrifuge preparations of sorted $CD3^+$,HNK1(−) lymphocytes (A), $CD3^+$,HNK1(+) lymphocytes (B) and $CD3^+$,HNK1(++) lymphocytes (C) after May–Grünwald–Giemsa staining. original magnification × 64. For description, see Results.

recall the study of Phillips and Lanier (1985), who have reported that a proportion of $CD16^+$,$HNK1^-$ lymphocytes acquire HNK1 after a 6-day stimulation with K562 erythroleukemia cells. Those authors have suggested that HNK1 expression is a late event in the differentiation of $CD16^+$ lymphocytes. An extrapolation of those combined observations to our study suggests that among the lymphocytes expressing HNK1, and particularly those which do so at high levels (HNK1(++)), are cells that have differentiated into CMV-specific cytotoxic lymphocytes. The relatively high frequencies of HNK1(++) lymphocytes in CMV-seropositive individuals (on average 1:18 lymphocytes) and even in CMV-seronegative individuals (1:40 lymphocytes) indicate that their specificities include other targets than CMV-infected cells.

The exact sites serving as reservoirs for CMV are unknown. *In vitro*, the binding of beta-2-microglobulin by CMV enhances its infectivity and allows the use of class I HLA molecules as viral receptors (Grundy *et al.*, 1987). This characteristic may result in a widespread dissemination of CMV in its carriers. We suggest that the increased numbers of granular lymphocytes expressing high levels of HNK1 reflect their involvement in keeping the replication of CMV in its carriers under control.

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