Immunology 1989 **69** 166–170

Heterogeneity of human natural killer cells in the spleen

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Accepted for publication 11 September 1989

SUMMARY

Natural killer cells have an important role in tumour and viral defence and immunoregulation. In the present study the pan-NK cell monoclonal antibody NKH1 was utilized to study the heterogeneity of NK cells in the human spleen. Using one and two colour analysis it could be demonstrated that the majority of $NKH1^+$ NK cells are located in the red pulp and marginal zone whereas only a minor subset is found in the lymph follicles. In the red pulp, NK cells resemble those of peripheral blood in terms of function and phenotype. In contrast, the few NK cells in the lymph follicles are mostly $NKH1^+$, $CD2^-$ and $CD16^-$ and thus express a unique phenotype. The analysis of tissue distribution provides a basis for further studies on NK cell kinetics and differentiation.

INTRODUCTION

Natural killer (NK) cells are a small heterogeneous lymphocyte subpopulation of about 10–15% in peripheral blood and are characterized by their ability to lyse tumour cells in a non-MHC restricted manner (Hercend & Schmidt, 1988). In addition to lysis of tumour targets and virally infected cells, regulation of the immune response (Vyakarnam *et al.*, 1985) and haematopoiesis (Herrmann *et al.*, 1987) appear to be major functions of NK cells. There is increasing evidence for a physiological role of NK cells in viral disease such as chronic Epstein–Barr virus (EBV) infection or the lympho-proliferative syndrome X (Caligiuri *et al.*, 1987). After *in vitro* and *in vivo* activation by interleukin-2, NK cells termed lymphokine-activated killer (LAK) cells are used in cancer therapy (Rosenberg *et al.*, 1985). There is little knowledge on the organ distribution and compartmentalization of human NK cells. Early studies of the distribution of NK cells in human tissues were possible when the HNK-1 (CD57) antibody had been described (Abo & Balch, 1981). The spleen appeared as an organ with a particular high frequency of HNK-1 $(CD57)^+$ cells. Ten to fifteen per cent of lymphocytes expressed the HNK-1 antigen (Ritchie, James & Micklem, 1983; Pizzolo *et al.*, 1984). Different phenotypes were observed in the red pulp and lymph follicles, where the majority of HNK-1 $(CD57)^+$ cells co-expressed CD4. This antigen is hardly present on any NK cells in peripheral blood or spleen (Pizzolo *et al.*, 1984).

Later the pan-NK specific antibody NKH1 (CD56) was generated and largely used together with CD16 antibodies to determine peripheral blood NK cells. It then became clear that HNK-1 was only reacting with a subpopulation of about 60% of NK cells. Moreover, there is cross-reactivity of HNK-1 with T and B cells (Hercend *et al.*, 1985; Abo, Cooper & Balch, 1982), and other cell types unrelated to the haematopoietic lineage (Bunn *et al.*, 1985; Schubert & Schmidt, 1989). NK cells are now defined as NKH1 $(CD56)^+$ $CD3^-$ cells. The NKH1 $(CD56)^+$ $CD3^+$ subset represents non-MHC-restricted T cells (Hercend & Schmidt, 1988).

In light of the increasing role of NK cells in pathophysiology of disease as well as in cytokine therapy, the knowledge of the distribution of this subpopulation of lymphocytes appears to be very important. The present studies were therefore undertaken to characterize the localization of distinct NK subsets in the human spleen. We examined especially numbers, function and heterogeneity in different splenic compartments in order to gain more information on differential function of human NK cells.

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MATERIALS AND METHODS

Tissue sources

Spleens were obtained from patients undergoing total gastrectomy ($n = 16$), patients with traumatic rupture of the spleen ($n = 3$), and from one patient with immune haemolysis. Single cell suspensions from spleens were prepared immediately after their removal. Peripheral blood was obtained from the same donors and peripheral blood lymphocytes (PBL) were prepared parallel to splenic lymphocyte (SL) suspensions. Fresh spleens were minced and mashed through a sieve. Lymphocytes were isolated from a Ficoll gradient. Cells in the interphase were washed three times in RPMI supplemented with 10% FCS.

Monoclonal antibodies

All monoclonal antibodies utilized in this study have been described previously in detail. CD2 (Leu 5; Becton-Dickinson, Heidelberg) defines an epitope on T and NK cells. 2AD2A2 (IgM), generously provided by Dr E. L. Reinherz, Dana Farber

Cancer Institute, Boston, MA, and Leu 4 (CD3; Becton-Dickinson, Heidelberg) define all mature T lymphocytes and a subset of NK cells. CD4 (OKT4; Ortho, Raritan, NJ) reacts with a subset of T cells with inducer/helper function. CD11a (2F12, IgG1) is a mAb against the alpha chain of the LFA-1 molecule (Schmidt *et al.*, 1985a). VEP-13 (IgM) and Leu 11a (Becton-Dickinson) are CD16 antibodies defining the Fcγ RIII receptor expressed on NK cells and granulocytes (Tetteroo *et al.*, 1987; Schmidt & Perussia, 1989). CD56 (NKH1 = Leu19; Becton-Dickinson) is expressed on all NK cells in peripheral blood (Hercend *et al.*, 1985; Schubert, Lanier & Schmidt, 1989a). CD57 (HNK-1 = Leu 7; IgM, Becton-Dickinson) is found on a subset of T and NK cells (Schubert *et al.*, 1989). The γ/δ T-cell receptor (anti-TcR delta 1; Hermann Biermann GmbH, Bad Nauheim) is expressed on a subset of T cells.

Phenotypic studies

Cell surface antigens were determined using direct and indirect immunofluorescence as previously described (Schmidt *et al.*, 1987). One and two colour analysis was performed by flow cytometry on a FACScan (Becton-Dickinson) counting 10,000 cells per sample.

Cytotoxicity assays

To assess NK activity 5000 ^{51}Cr-labelled K562 target cells were incubated with various numbers of effector cells at 37° in V-bottomed microtitre plates as described previously (Schmidt *et al.*, 1985b; Werfel *et al.*, 1989). RPMI-1640 was medium supplemented with 5% fetal calf serum (FCS) and 1% penicillin-streptomycin. Cytotoxicity was measured by release of ^{51}Cr into the supernatant after 4 hr incubation. Maximal release was determined by addition of 1% Triton-X-100 (Sigma, Poole, Dorset, U.K.). Spontaneous release was < 10%. Specific release was calculated as follows:

$$\frac{\text{mean experimental c.p.m.} - \text{mean spontaneous c.p.m.}}{\text{mean total c.p.m.} - \text{mean spontaneous release}}.$$

Immunohistology

Pieces of the spleens were washed in RPMI-1640, snap-frozen in liquid nitrogen, cut on a cryostat in 4–8 μm sections, air dried for 2 hr and stored at −20° until staining.

The APAAP technique was applied according to a method described by Cordell *et al.* (1984). Primary antibodies were used in a dilution of 1:100.

In double immunofluorescence studies, NK cells were investigated with Leu 19 PE (direct) at a dilution of 1:6 and applied with indirect antibodies of IgM isotope (2AD2A2 = CD3, VEP13 = CD16, HNK-1) at a dilution of 1:40 or with Leu 5b FITC (CD2) at 1:6. Goat anti-mouse IgM-FITC (1:15) was used as secondary antibody. The sections were evaluated using a fluorescence microscope (Leitz Orthomat, Wetzlar) equipped with selective filters for the fluorochromes used. In each splenic compartment, 100 cells were counted and evaluated for the expression of the antigens examined.

RESULTS

Lymphocyte subpopulations in blood and spleen

Splenic cell suspensions and peripheral blood of 20 patients were analysed using single and double immunofluorescence. Twenty-five (±9)% of splenic lymphocytes were identified as NKH1 (CD56)$^+$ NK cells. Fifty (±10)% of the lymphocytes expressed CD3$^+$ and 33 (±9)% CD20$^+$ (Fig. 1). Seventy per cent of the NKH1$^+$ cells co-expressed CD16, 30% CD3 and 80% CD2. As 8% of splenic lymphocytes were NKH1$^+$ CD3$^+$ T cells, the percentage of NK cells in the spleen was 17%. Four per cent of splenic lymphocytes expressed the γ/δ T-cell receptor.

When comparing the number of NK cells in peripheral blood it was always found to be lower than in the spleen, but the antigen distribution on these cells was similar. Eighteen (±5)% of the PBL were CD56$^+$, 70% of these cells co-expressed CD16, 30% CD3, and 82% CD2 (data not shown).

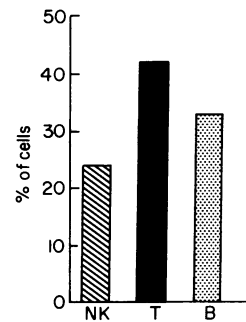

Figure 1. Splenic lymphocyte subpopulations. The percentage of NK cells NKH1$^+$ (▨), T cells CD3$^+$ (■) and B cells CD20$^+$ (▩) was determined using FACS analysis of splenic lymphocyte suspensions as described in the Materials and Methods.

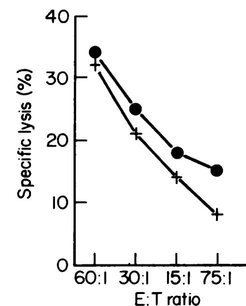

Figure 2. Cytotoxicity of splenic (+) and blood (●) NK cells. K562 killing was measured at various E:T ratios as described in the Materials and Methods.

Cytotoxic activity of splenic and peripheral blood lymphocytes

In order to compare the function of NK cells in blood and spleen their cytotoxic activity was determined. At an E:T ratio of 60:1 specific lysis of SL was 34% compared to 32% for PBL (Fig. 2). Cytotoxic activity of SL and PBL was similar although low compared to normal donors' PBL.

Compartmentalization of NK cells in the spleen

To localize NK subpopulations within the spleen the distribution of NKH1 (CD56)$^+$ cells was examined by immunohistology. In the red pulp and marginal zone many scattered CD56$^+$ cells were stained (Fig. 3). Control sections revealed no background staining demonstrating the specificity of this

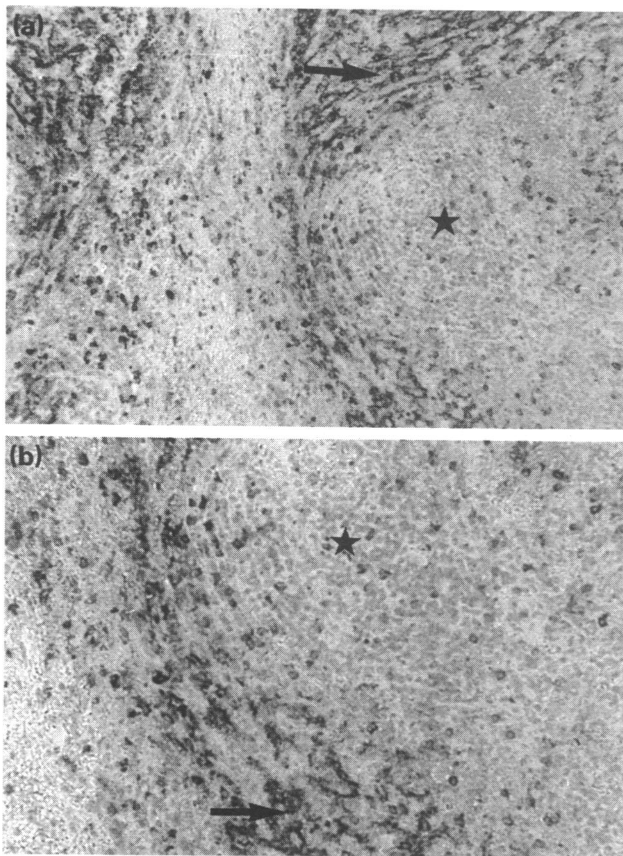

Figure 3. (a) NKH1$^+$ cells in the spleen. Follicles (*) where characteristically only a few NK cells are detected. In the red pulp many NKH1$^+$ cells are stained (↑). (b) The boundary of red pulp and follicle from (a) in larger magnification.

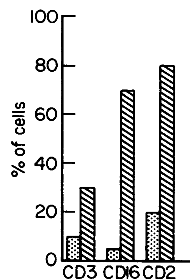

Figure 4. Co-expression of CD2, CD3 and CD16 on NKH1$^+$ NK cells in red pulp (▧) and lymph follicles (▩). Two colour analysis was performed on a FACScan counting 10,000 cells as described in the Materials and Methods.

method. NKH1 (CD56)$^+$ cells represented a proportion of approximately 30% of lymphocytes in this compartment. In lymph follicles few NKH1$^+$ cells could be detected, representing only 1–3% of the lymphocytes here.

As remarkable differences in the distribution of NK cells in the red pulp and lymph follicles could be shown we were interested as to whether the compartmentalized NK cells represented phenotypically distinct subsets. The co-expression of NKH1 with various antigens in the different splenic compartments was studied (Fig. 4).

Significant differences could be demonstrated between NK cells in red pulp and lymph follicles. In the red pulp and marginal zone 70% of NKH1 NK cells were positive for CD16, 30% for CD3 and 80% for CD2. Of the NK cells in the lymph follicles, however, only 5% were CD16$^+$, 10% CD3$^+$, and 20% CD2$^+$ (Fig. 4).

CD4 was not co-expressed with NKH1 on cells from the red pulp or the lymph follicles as described previously for HNK-1 (Pizzolo *et al.*, 1984). However, in the lymph follicles NKH1$^+$ cells did not co-express HNK-1 either whereas in the red pulp 60% of NKH1$^+$ cells were HNK-1$^+$ (data not shown). This suggests that the HNK-1$^+$ and NKH1$^+$ cells in the lymph follicles represent different subsets. Co-expression of CD8 was similar on NK cells in the red pulp and the lymph follicles. Forty per cent of NHK1$^+$ cells in each compartment were CD8$^+$ (data not shown). Both the NKH1$^+$ CD3$^+$ and the γ/δ^+ T-cell subset were located mainly in the red pulp and the periarteriolar lymphocyte sheaths.

DISCUSSION

The present studies addressed the distribution, heterogeneity and function of NK cells in human spleen. It has been demonstrated that NK cells play an important role in the defence against human tumours as well as in therapy of human malignancies using 'so called' LAK cells or cytokines. In addition phenotypic and functional changes of NK cells have been observed during various viral diseases especially in chronic EBV disease. In addition there is increasing evidence for a regulatory role of NK cells in haematopoiesis or of the immune system itself, such as in immunoglobulin production.

The development of NK-specific antibodies has helped to analyse the role of NK cells and their surface molecules. A number of recent reports have accumulated information on the distribution of NK cells in human peripheral blood and the tremendous heterogeneity in this population of lymphocytes. It could be demonstrated clearly that within the population of NKH1$^+$ NK cells, which represent about 15% of peripheral blood lymphocytes of normal donors, about 75% are CD3$^-$ and CD16$^+$. In addition, 25% of NKH1$^+$ cells co-express CD3 antigen but exert non-MHC restricted cytotoxicity and should be regarded as T cells. Again some of these NKH1$^+$ CD3$^+$ cells co-express CD16 whereas about 2·5% of NKH1$^+$ CD3$^-$ cells are negative for the CD16 antigen (Lanier *et al.*, 1986; Ritz *et al.*, 1988).

Our data on NK cells in normal human spleen identify a significantly higher percentage of NKH1$^+$ NK cells (mean = 25%) than in peripheral blood. The co-expression of other cell surface antigens such as CD16, CD3 or CD2 is very similar, as has been described for peripheral blood NK cells.

These results are in agreement with reports on the distribution of mouse NK cells where the majority of NK cells have always been attributed to the spleen (Tam, Emmons & Pollack, 1980). The few studies performed in humans using the HNK-1 antibody did not give detailed numbers on HNK-1$^+$ lymphocytes in the total spleen cells. From our data it can be concluded that the majority of NK activity even in humans has to be localized in the spleen.

When analysing the function of these splenic NK cells the activity was quite comparable to the NK cells killing of

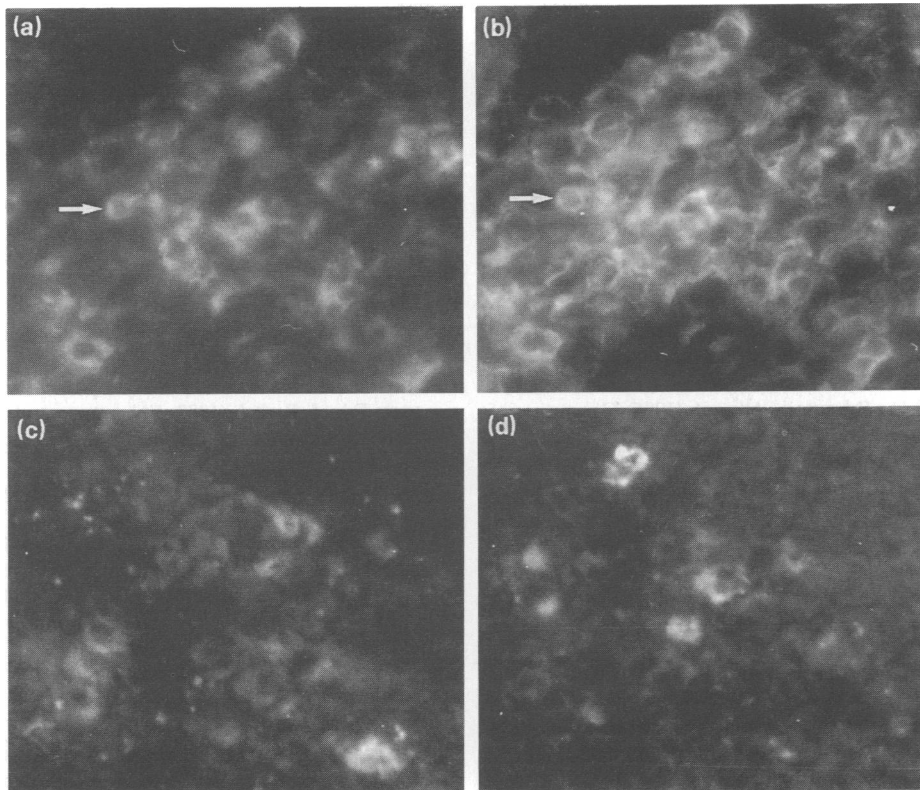

Figure 5. Double immunofluorescence studies of NK cells in the spleen. (a) NKH1 in red pulp; (b) the same area with CD2; (c) NKH1 in a follicle; (d) the same area with CD2. All NKH1$^+$ cells in (a) co-express CD2. (↑) Indicates a NKH1$^+$ CD2$^+$ cell. In (c) and (d) all NKH1$^+$ cells are CD2$^-$.

peripheral blood lymphocytes of the same donors. Taking into consideration that the relative percentage of NK cells was higher in spleen, lower functional activity than in blood has to be assumed.

In earlier studies (Pizzolo *et al.*, 1984) a very low percentage of HNK-1$^+$ lymphocytes within the germinal centres has been described. Comparing the red pulp and marginal zones for the presence of NKH1$^+$ cells we were able to confirm these previous data. Some of the staining observed in the immunohistochemical studies may be due to staining of NKH1 (CD56) antigen expressed on autonomous nerve fibres in the spleen since NKH1 is identical to the 140,000 MW isoform of human neuronal cellular adhesion molecule (N-CAM) (Lanier *et al.*, 1989).

Only 2% of spleen cells in the germinal centres expressed NKH1 antigen. Further analysis revealed that these NK cells were phenotypically quite different from those in the red pulp. As shown in Figure 4 almost all NK cells in the lymph follicles were CD16- and CD2-negative. The observation of this very small but phenotypically quite interesting subset raises the question of whether these CD16$^-$ CD2$^-$ NKH1$^+$ NK cells might have lost the cells surface antigens by emigrating into the lymph follicle, although this process, at least in the rat, appears to occur only rarely (Rolstad, Herberman & Reynolds, 1986). It also could potentially be an early progenitor cell for peripheral blood NK cells. Further studies are necessary to clone the cells from this particular subset of NK cells after purification and study their differentiation potential with various cytokines.

In summary, these data on the distribution of human NK cells will help to understand further the tremendous changes of peripheral blood NK cells in clinical situations such as viral diseases or cytokine treatment. The high numbers of NKH1 (CD56)$^+$ cells within the spleen could be a reservoir for NK cells being mobilized and activated after *in vivo* IL-2 or interferon-alpha treatment. A subset of CD56$^+$ CD16$^-$ NK cells was observed recently by our group after IL-2 treatment (Schubert *et al.*, manuscript submitted). Conversely, in early phases of IL-2 treatment, or as recently described for tumour necrosis factor alpha, these cells could emigrate from peripheral blood and adhere in the red pulp of the human spleen (Kist *et al.*, 1988). The data presented here provide a further basis for studying these questions.

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeineschaft Schm 596/2-1 and 596/2-2. We wish to thank Professor Meyer and Professor Reilmann, Department of Surgery, and their colleagues, who let us study the spleens. We are grateful to Dr Westermann for critical discussion and Mrs E. Schürmann for excellent typing.

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