Effects of sera from patients with Hodgkin's disease and of cytokines on the expression of parallel tubular structures and cell surface antigens in lymphocytes
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DISCUSSION AND CONCLUSIONS

The key terms which form the basis of this thesis are Hodgkin's disease (HD), granular lymphocytes, parallel tubular structures (PTS), induction, serum factor(s), interferon-α (IFNα), immunoelectron microscopy (IEM), and natural killer (NK) activity.

The peripheral blood of patients with newly diagnosed, untreated HD contains a than normal higher percentage of lymphocytes containing PTS and/or electron dense granules in their cytoplasm (granular lymphocytes). Serum from certain untreated HD patients demonstrate the property of being able to induce (in vitro) an increase in the percentage of granular lymphocytes and a concomitant augmentation of NK activity in peripheral blood mononuclear cells (PBMC) from healthy donors.

The main aims of the present investigation were to reveal the identity of the unknown factor responsible for the granular lymphocyte induction and NK activity enhancement, determine the subpopulations(s) to which the granular cells belong, and gain insight into the function of these cells in HD patients. Five out of seven chapters are directly concerned with these objectives, whereas two indirectly. Of the latter, one is a technical note regarding probe choice in IEM, while the other is a morphological study concerning the incubation effects on cells containing the morphologically distinct PTS.

The general introduction is presented in CHAPTER 1. Besides the above mentioned aims of the investigation, this chapter encompasses a short review on the lymphocytes implicated in NK cell cytotoxicity, including their morphological and phenotypical characteristics, some aspects of their regulation and their relationship to malignant disease.

In CHAPTER 2 we report the induction of granular lymphocytes after incubation of healthy donor PBMC in the presence of natural IFNα (highly purified). NK activity is known to reside in a proportion of lymphocytes with the morphological characteristics of large granular lymphocytes (LGL). IFNα is known to enhance NK activity. This property of IFNα prompted us to
examine its effect(s) on PBMC, and particularly on granular lymphocytes. At the time of commencement of this study, only the naturally produced form of IFNα, and not the recombinant product, was commercially available. Surface antigen reactivity with the monoclonal antibodies (mAb) detecting CD 3 (OKT 3) (T cells), CD 4 (OKT 4) (helper/inducer T cells), CD 8 (OKT 8) (cytotoxic/suppressor T cells), and CD 57 (Leu-7) (NK cells/T cell subset) in IEM (single immunogold staining) revealed no differences to exist between nIFNα-treated and control preparations. However, significant increases in the percentages of granular cells within the CD 8⁺- and CD 57⁺-lymphocyte populations after nIFNα incubation were observed. The highest increase of granular cells was within the CD 8⁺-cells. In addition, IFNα-treated PBMC exhibited augmented NK cytotoxicity. IEM demonstrated that nIFNα mainly induces an increase in granular cells which express CD 8. A minority express CD 57. It is also possible that cells coexpress CD 8 and CD 57. Although mAb detecting CD 8 mainly defines cytotoxic/inducer T cells, these findings suggest a possible role for CD 8⁺-granular cells in the nIFNα-enhanced NK cytotoxicity.

In CHAPTER 3 a double immunogold labeling method in IEM which allows for the simultaneous detection of two lymphocyte surface antigens, with the preservation of morphology, is described. Elaboration of the markers applied in chapter 2 with a mAb detecting NK cells, Anti-Leu 11b (CD 16), and using this marker in combination with CD 8 to further characterize the induced granular cells in chapter 2, led to the finding that the combination of gold probe size (5nm or 15nm) and mAb decisive is for detecting double labeled cells with the CD 16⁺,-8⁺ phenotype. Determination of antigen density by means of immunofluorescence and fluorescent activated cell sorter (FACS) analysis revealed the antigen density of CD 8 on CD 16⁺,-8⁺ lymphocytes to be low. IEM demonstrated that when the 15nm gold probe was applied to detect CD 8 and the 5nm gold probe to detect CD 16, no double labeling occurs. The reverse combination resulted in double labeled CD 16⁺,-8⁺ lymphocytes. For CD 57 and CD 8, double labeled cells were detected irrespective of which gold probe combination was applied. The CD 8 antigen density on CD 57⁺ cells is higher than on CD phenotypical than on CD 57⁺ cells.

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lymphocytes. The double labeling in the immunogold technique and implies possible functional heterogeneity. From the labeling findings we concluded that although the double immunogold labeling technique is an excellent method to study the coexpression of surface membrane antigens of morphologically identifiable lymphocytes, it is important to determine suitable combinations of gold probes and mAbs to ensure that lymphocytes are not falsely perceived as negative for a particular mAb combination.

In CHAPTER 4 the double labeling combinations tested in chapter 3 were utilized to determine the surface markers on the by HD serum induced granular lymphocytes. Application of surface markers detecting CD 16, CD 57 and CD 8 revealed that a significantly large proportion of the granular cells expressed CD 16 (CD 16+/PTS+), while only minor proportions were positive for CD 57 and CD 8. Of the double labeled lymphocytes, only those coexpressing CD 16 and CD 8 (CD 16+,8+/PTS+) were increased with respect to the control (human pooled serum) incubation. Assessment of the total percentage of mAb+ cells (i.e. without taking into account the morphology), revealed only the percentage of CD 16+ lymphocytes to be significantly increased with HD serum. Furthermore, HD serum incubation augmented effector cytotoxicity against the K562 tumor cell line.

An unknown factor (or factors) present in certain HD sera - inducing (I)-sera - appears to be responsible for the observed morphological, phenotypical and functional changes. Sera subjected to temperature changes and to acid pH demonstrated resistance to this treatment in that the capacity to induce an increase in granular lymphocytes was retained.

On the basis of these observations it was concluded that a cytokine (or a combination of cytokines) affecting lymphocytes with the potential of becoming cytotoxic, may be involved in the effects demonstrated by HD I-sera. IFNo, in particular, may be a possible candidate considering the comparable, although not identical (with nIFNo a larger increase in CD 6+/PTS+ cells was observed), findings reported in chapter 2.
In CHAPTER 5 the membrane characteristics of nIFNα induced lymphocytes (described in chapter 2) were further delineated. Besides elaboration with the mAb detecting CD 16, double labeling combinations were also investigated by means of the immunophenotyping such as described in chapter 3. The greatest proportion of granular lymphocytes expressed CD 16 (CD 16⁺), with lesser proportions positive for CD 8, CD 57, or coexpressing CD 16 and CD 8 (CD 16⁺,-8⁺/PTS⁺). The results obtained with nIFNα resemble those obtained with the HD I-serum incubation (chapter 4).

It must be mentioned, however, that the results obtained with CD 8 (OKT 8) in the present chapter and in chapter 2 are slightly divergent. In chapter 2 there is quite a substantial increase in the percentage of CD 8⁺-granular lymphocytes. This prompted us to hypothesize that the induced granular cells may coexpress CD 16 and CD 8. Although a significant increase of the latter cells was observed in the current chapter, it was not of the magnitude predicted. At this moment we can only speculate on why this should be so. The natural IFNs used in the experiments in chapters 2 and 5, although both highly purified and from the same manufacturer (Sigma), were supplied in different forms (one lyophilized and the other in solution). The method of production may have influenced the biological activities of these products. In the first study (chapter 2) we applied 500 U/ml whereas in the present study the concentration was 1000 U/ml. Both these concentrations were applied after testing with PBMC from various donors to determine the concentration which gave the most consistent results. Unfortunately the product originally used remains unavailable. In control studies the substitution of OKT 8 (Ortho Diagnostics) monoclonal antibody by anti-Leu 2 (Becton Dickinson) did not affect the outcome of the labeling.

On the basis of the morphological characteristics and the surface antigen expression displayed by the granular lymphocytes and the augmentation of NK activity after nIFNα induction, it can be concluded it is highly likely that these granular lymphocytes are functional NK cells.

Comparable incubations with the recombinant form of IFNα (rIFNα) and both the natural and recombinant forms of gamma-IFN (IFNγ) resulted in increases in NK activity but, in contrast to nIFNα, not the concomitant increase of the morphological characteristics of the induced granular cells observed with nIFNα. This may indicate different effects of the two IFNs on the pathways of NK cell activation.

In the present study, by increasing the mAbs detecting the morphological differentiations, we could show that the majority of induced granular lymphocytes coexpressed CD 16 and CD 8. This was not the case for the majority of cells coexpressing CD 16 and CD 8. Although a significant increase of the latter cells was observed in the current chapter, it was not of the magnitude predicted. At this moment we can only speculate on why this should be so. The natural IFNs used in the experiments in chapters 2 and 5, although both highly purified and from the same manufacturer (Sigma), were supplied in different forms (one lyophilized and the other in solution). The method of production may have influenced the biological activities of these products. In the first study (chapter 2) we applied 500 U/ml whereas in the present study the concentration was 1000 U/ml. Both these concentrations were applied after testing with PBMC from various donors to determine the concentration which gave the most consistent results. Unfortunately the product originally used remains unavailable. In control studies the substitution of OKT 8 (Ortho Diagnostics) monoclonal antibody by anti-Leu 2 (Becton Dickinson) did not affect the outcome of the labeling.

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lymphocytes also resemble CD 8 (OKT T) cells. In a recent study, it was shown that the induced \( \text{IFN}\gamma \) is of a difference species to r\( \text{IFN}\alpha \), which could explain the discrepancy between the cytotoxic and morphological events. However, the same is not true for r\( \text{IFN}\alpha \). The difference lies in the method of production which could influence the molecular structure of r\( \text{IFN}\alpha \) and thereby also its mode of action. In all, this suggests that, even though the difference IFNs applied have comparable effects on the cytotoxic function of effector cells, the mechanisms or pathways by which cytotoxicity is achieved are apparently not identical considering the observed morphological discrepancy.

CHAPTER 6 compares the phenotypical and ultrastructural characteristics of freshly isolated and incubated lymphocytes containing the actual PTS (PTS with or without electron dense granules) to those containing only the electron dense granules. The results showed that under all conditions the majority of CD 16\(^+\) lymphocytes (64.3–74.8%) contained PTS, whereas these structures were observed in 41.8% CD 57\(^+\) lymphocytes. In the case of cells coexpressing CD 16 and CD 8 (CD 16\(^+\),-8\(^+\)), 41.0–61.7% contained PTS, but only 24.2–27.5% CD 57\(^+\),-8\(^+\) cells were PTS\(^+\). These results indicate PTS to be a more distinctive morphological marker for CD 16\(^+\) than for CD 57\(^+\) and CD 8\(^+\) lymphocytes. Additionally, it was observed that although the PTS\(^+\) cells exhibited phenotypic heterogeneity, they generally exhibited morphological homogeneity. Only a proportion of CD 57\(^+\),-8\(^+\) cells exhibited the morphological features of T cells. As CD 16\(^+\) lymphocytes are potentially more cytotoxic than both CD 57 and CD 8 lymphocytes, it may be concluded that the morphologically distinct PTS play an important role in target cell destruction.

In the investigation described in CHAPTER 7, analysis of the lymphocytes present in the peripheral blood of patients with HD using the mAbs detecting CD 16, CD 57 and CD 8 in IEM, revealed patients to contain increased percentages of CD 16\(^+\) and CD 57\(^+\) lymphocytes compared to healthy donors. No difference was found in mean NK activity between HD patients and healthy donors. However, NK activity levels varied widely among the HD patients. Subdividing the patients into two main groups according to...
whether their NK activity was greater than mean control (group I) or less than or equal to mean control (group II), revealed that the group I patients contained higher percentages of total CD 16\(^+\) and CD 16\(^-\)-granular cells than the group II patients, whereas group II patients contained higher percentages of total CD 57\(^+\) and CD 57\(^-\)-granular cells. Thus, the PBMC of the group I patients contained higher levels of potentially more cytotoxic lymphocytes than those in group II patients. Furthermore, the sera of 5 out of 6 group I patients were sera exhibiting the capacity to induce an increase in granular lymphocytes and augment NK activity (i.e. inducing-sera as described in chapter 4) whereas all of the group II sera tested were of the non-inducing type (NI-sera). These results suggest a positive relationship to exist between the unknown inducing factor present in a HD serum, the NK activity of the patient lymphocytes from whom the I-serum was obtained, and the phenotypic characteristics of the granular lymphocytes in HD.

The effects of applying neutralizing antibodies against IFN to a HD I-serum are described in CHAPTER 8. From the comparable results obtained in chapters 4 and 5, it seemed plausible to postulate an instrumental role for IFN\(_\alpha\) in HD I-sera in bringing about the increase, or at least a proportion of the increase, in granular lymphocytes and augmentation of cytotoxicity. Preincubation of I-sera with anti-IFN\(_\alpha\) resulted in negation of the previously observed effects. This was not the case upon application of the antibodies against IFN\(_\beta\) and IFN\(_\gamma\): with the latter two the inducing effects were retained.

The discrepancy observed between nIFN\(_\alpha\) and rIFN\(_\alpha\) with respect to the induction of granular cells, as described in chapter 5, prompted us to investigate whether rIFN\(_\alpha\) lacked an essential "natural" factor. For this purpose NI-serum and rIFN\(_\alpha\) were incubated simultaneously with healthy donor lymphocytes. The outcome was an increase in the percentage of granular lymphocytes. This effect could again be negated by anti-IFN\(_\alpha\), which indicates that an IFN\(_\alpha\) could most certainly be necessary for the induction. Other cytokines applied simultaneously with rIFN\(_\alpha\) could not reproduce the effect. The factor or mechanism involved in the synergism between rIFN\(_\alpha\) and
hat the group I patients contained cells. Thus, the potentially more group II sera results suggest a factor present from whom the I-s of the granular lymphocytes express the CD 16 differentiation antigen (i.e. anti-IFNo, which for the induction. Concluding remarks

This thesis shows that the greatest proportion of the induced granular lymphocytes express the CD 16 differentiation antigen and exhibit the morphological characteristics of functional NK cells.

From our experiments it appears probable that the previously unknown factor, present in sera from certain patients with HD and responsible for the induction of granular lymphocytes, can be viewed in relation to IFNo. The effect of incubating PBMC with nIFNo was comparable to that obtained with HD serum. Although elevated levels of IFNo have not been detected in HD sera, application of antibodies against IFNo to a I-serum blocks the inducing effect.

Untreated HD patients exhibit a higher percentage of LGL in their peripheral blood than healthy persons. This suggests a stimulatory or inductive mechanism affecting certain lymphocytes in HD. The answer to the question whether IFNo is directly responsible for the increased proportion of granular lymphocytes in HD patients, or whether it is an integral part of a multiple-step of mechanism, is not known. Furthermore, if IFNo contributes to the level and function of granular cells in HD patients, this probably varies among individual patients. Irrespective of stage of disease or histopathology, the sera from the majority of patients with higher than mean control NK activity exhibited granular lymphocyte inducing capacity, and their peripheral blood exhibited elevated levels of both granular and agranular CD 16 lymphocytes. All sera from patients with normal or lower than normal NK activity were, on the otherhand, non-

NI-serum remains elusive. Nonetheless, the results implicate IFNo as the unknown factor present in I-serum responsible for granular lymphocyte induction and augmentation of NK activity.

It is also possible that circulating factors derived from the tumor in HD affect the patient lymphocytes. However, in preliminary experiments whereby supernatants from various established Hodgkin cell line cultures were applied to PBMC from healthy donors, neither an increase in granular cells nor augmentation of cytotoxicity were observed. This suggests once more that the inducing factor is a not a tumor cell product.
inducing and the percentage of CD 57+ cells higher. Therefore, although in most patients with HD the proportion of granular cells is increased, these cells do not have the same phenotype and consequently not the same function. CD 16+ lymphocytes have the potential of becoming highly cytotoxic, more so than CD 57+. These findings could be important in relation to the progression of the disease. The causes responsible for the differences observed between the individual HD patients need to be resolved in further studies.