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Bystander Cells and Prognosis in Hodgkin Lymphoma

Review based on a doctoral thesis

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ABSTRACT

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Hodgkin lymphoma (HL) is characterised histologically by a minority of malignant Hodgkin and Reed-Sternberg (HRS) cells surrounded by benign cells, and clinically by a relatively good prognosis. The treatment, however, leads to a risk of serious side effects. Knowledge about the biology of the disease, particularly the interaction between the HRS cells and the surrounding cells, is essential in order to improve diagnosis and treatment.

HL patients with abundant eosinophils in the tumours have a poor prognosis, therefore the eosinophil derived protein eosinophil cationic protein (ECP) was studied. Serum-ECP (S-ECP) was elevated in most HL patients. It correlated to number of tumour eosinophils, nodular sclerosis (NS) histology, and the negative prognostic factors high erythrocyte sedimentation rate (ESR) and blood leukocyte count (WBC). A polymorphism in the ECP gene (434(G>C)) was identified and the 434GG genotype correlated to NS histology and high ESR.

The poor prognosis in patients with abundant eosinophils in the tumours has been proposed to depend on HRS cell stimulation by the eosinophils via a CD30 ligand (CD30L)-CD30 interaction. However, CD30L mRNA and protein were detected in mast cells and the predominant CD30L expressing cell in HL is the mast cell. Mast cells were shown to stimulate HRS cell lines via CD30L-CD30 interaction. The number of mast cells in HL tumours correlated to worse relapse-free survival, NS histology, high WBC, and low blood haemoglobin.

Survival in patients with early and intermediate stage HL, diagnosed between 1985 and 1992, was generally favourable and comparatively limited treatment was sufficient to produce acceptable results for most stages. The majority of relapses could be salvaged. Patients treated with a short course of chemotherapy and radiotherapy had an excellent outcome.

In conclusion prognosis is favourable in early and intermediate stages and there

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are possibilities for further improvements based on the fact that mast cells and eosinophils affect the biology and prognosis of HL.

INTRODUCTION

Thomas Hodgkin and the early reports of the disease

Thomas Hodgkin was born in 1798 in Tottenham, Middlesex, England in a family of Quakers. He was educated at the University of Edinburgh, as he was unable to study at Oxford or Cambridge due to his Quaker background. In 1832 he published the first article on malignant lymphomas called "On some morbid appearances of the absorbent glands and spleen" (1), where he described seven patients with generalised lymphadenopathy and splenomegaly examined post mortem. At the time Thomas Hodgkin made these initial studies he was a Curator of the Museum and Inspector of the Dead at Guy's hospital in London (2, 3). It was also known in the 19th century that enlarged lymph nodes could be caused by for example cancer or tuberculosis. However, Hodgkin suggested that the disease in some cases could start *de novo* in the lymph nodes, not being the result of disease somewhere else in the body. Indeed, the earliest known description of the appearance of enlarged lymph nodes and spleens dates back to 1669, written by Malpighi from Bologna (4), after his discovery of the blood capillaries in 1661 (2).

Material from four of the cases T. Hodgkin described were preserved at the museum at Guy's hospital and later re-examined. Two of the four cases were indeed Hodgkin lymphoma, one probably another kind of lymphoma, and the last one an inflammatory lesion, possibly tuberculosis (2, 5, 6). Considering the later development in diagnosis of malignant lymphomas and the never-ending story of lymphoma classifications, 50% correct diagnoses must be considered a high percentage, as these diagnoses were made before the disease was even named, and before microscopy was available.

Important accomplishments by T. Hodgkin besides describing lymphomas were, to name a few, the introduction of the stethoscope in Great Britain, pathologically describing perforated appendicitis and local spread of cancer, describing aortic insufficiency, recommending more fiber and less sugar and meat in the diet, recognising the contagious nature of cholera, and suggesting improvements in the situation of the poor to prevent spreading of the disease (2, 3). Later, when T Hodgkin failed to get an appointment as consultant at Guy's hospital, after a controversy with Benjamin Harrison, the hospitals treasurer, his interest turned to philanthropic activities. For example he founded the "Aborigines' protection society" (2, 3). When he visited the Holy Land with his friend Sir Moses Montefiore, he was struck by dysentery. He died in Jaffa in 1866 and his grave is there to be visited by those wishing to honour this man whose name will always be associated with malignant diseases in the lymphoid system. On his gravestone the words "Humani nihil a se alienum putabat" ("Nothing of humanity was foreign to him") are inscribed (2, 3).

Why the disease changes name in the thesis

The disease was named Hodgkin's disease in 1865 by Sir Samuel Wilks (7), 13 years before the first histopathological description was published by Greenfield (8), and 33 years after Thomas Hodgkin's article was published. However, with some exceptions in this thesis I have chosen to refer to the disease as Hodgkin lymphoma (HL) (or Hodgkin's lymphoma), which is more appropriate nowadays, when we consider HL to be a lymphoma among others rather than distinguishing between Hodgkin's disease and Non-Hodgkin lymphomas (NHL) as was done in the past. In the new World Health Organisation (WHO) classification the disease is also referred to as HL (9).

Dorothy Reed and Carl Sternberg describing the malignant cell

There are two other people who will always be associated with this disease, namely Carl Sternberg (1872–1935) and Dorothy Reed (1874–1964). They have given their names to the Hodgkin and Reed-Sternberg (HRS) cells. These cells are indeed the malignant cells of HL, however they are in a minority in the tumours, a fact that will be further explored in this thesis.

Carl Sternberg was an Austrian pathologist who in 1898 described large cells characteristic for HL, however comprising a minority of the cells in the tumours (10). His description of the HRS cells was further refined by Dorothy Reed, who in 1902 published the article "On the pathological changes in Hodgkin's disease, with special reference to it's relation to tuberculosis" (11) where she made clear that HL was not a variant of tuberculosis, and provided skilful drawings of the HRS cells. Previously the high proportion of tuberculosis in HL patients had lead pathologists to assume that HL might be a variant of tuberculosis. However, Reed showed that there was no reaction to tuberculin (11).

The never-ending story of lymphoma classifications

According to legend only three things in life are certain: that we will die, that we have to pay taxes, and that the lymphoma classification will change. Whether this is correct or not, the fact is that the classification of lymphomas have changed a number of times and that the frequency of these changes does not seem to diminish over time, as we shall see.

The first recognized attempt to classify lymphoproliferative diseases was made by Rosenthal in 1936 (12). He divided HL into four different types, depending on the degree of lymphocytic infiltration. This classification also had prognostic significance. The next classification, by Jackson and Parker came 11 years later in 1947 and described the three groups paragranuloma, granuloma, and sarcoma (13). This was modified nine years later, when another group of granuloma with more marked fibrosis and better prognosis was described (14). Another 10 years passed and the time came for a new classification. In this new classification a great effort was made to correlate the known microscopic features of the disease to prognosis. The result was a division into six different groups (15). From this the Rye classifi-

Table 1. HL classification, from the WHO classification of tumours of haematopoietic and lymphoid tissues (9).

Histology	Abbreviation	Short description	Frequency
Nodular lymphocyte predominant Hodgkin lymphoma	NLPHL	Usually CD30 negative; CD45, CD20 positive. Popcorn or L&H cells. Often localised, good prognosis.	5%
Classical Hodgkin lymphoma	CHL	CD30 positive, CD45 negative, CD20 positive or negative. HRS cells. Variants below:	95%
Nodular sclerosis CHL	NSHL	Collagen bands surrounding at least one nodule. Lacunar type HRS cells. Often numerous eosinophils. Relatively good prognosis.	70% of CHL
Mixed cellularity CHL	MCHL	Scattered HRS cells in a diffuse or vaguely nodular inflammatory background without nodular sclerosing fibrosis. Before modern therapy worse prognosis than NSHL, now comparable.	20-25% of CHL
Lymphocyte-rich CHL	LRCHL	Scattered HRS cells in nodular or diffuse background with an abundance of small lymphocytes. Absence of neutrophils and eosinophils. Relatively good prognosis.	5% of CHL
Lymphocyte-depleted CHL	LDHL	Rich in HRS cells and/or depleted in non-neoplastic lymphocytes. Often advanced stage, B-symptoms. Before modern therapy worse prognosis. Now very rare.	<5% of CHL

cation, which divides HL into four different groups, was developed (16, 17). The groups according to the Rye classification are: lymphocytic predominance (LP), nodular sclerosis (NS), mixed cellularity (MC), and lymphocytic depletion (LD). This classification was used for quite a long time; the next classification was published 28 years later (18). In the revised European-American lymphoma (REAL) classification both clinical and histopathological, as well as new immunophenotypic, cytogenetic and molecular knowledge is taken into account. Classical HL (CHL) is divided into lymphocyte-rich classical HL (LRCHL) (provisional), NS, MC, and LD. CHL is separated from nodular lymphocyte predominance (NLPHL) (18).

However, with the REAL classification only six years passed before the WHO classification emerged (9). In this classification (table 1), as in the REAL classification, CHL is separated from NLPHL, the histological groups are the same, but LRCHL is no longer a provisional entity. The WHO classification is currently used by haematopathologists, but from past experience, we know that one day it will be changed. The LD subgroup is today very rare as many cases previously diagnosed as LD are now recognised as lymphomas other than HL, with anaplastic or pleomorphic large cell morphology (9, 19).

The tumour cell

The HRS cells comprise only 0.1–10%, usually less than 3%, of the cell population in CHL tumours. Morphologically these cells are large, 20–25 μm, and usually have two separate nuclear lobes with prominent eosinophilic nucleoli and pale chromatin. The cytoplasm is slightly basophilic (20). In NLPHL the tumour cells are called popcorn or lymphocytic and histiocytic (L&H) cells. This histologic type is not discussed in detail in this thesis.

The origin of the HRS cells is usually germinal centre derived B-cells (21–23), however, in rare cases they can be T-cell derived (23–25). The most accurate way of studying the HRS cells is by single cell analysis. In studies of this kind presence of somatic hypermutations of the immunoglobulin (Ig) gene and simultaneous lack of Ig mRNA transcripts have been demonstrated. This is regardless of presence or absence of crippling Ig gene mutations caused by an inactivation of the Ig promotor. Down-regulation of the octamer-dependent transcription factor Oct2, its coactivator BOB.1, and the transcription factor PU.1 are suggested as reasons for this (23, 26, 27). The subsequent incapacity of HRS cells to produce Ig should lead to apoptosis, as is the case for normal B-cells that loose their capacity to express Ig (28). However, in some way they escape apoptosis.

Different ways for the HRS cells to escape apoptosis have been suggested, such as constant NFκB activation (23, 29, 30). This activation can for example be caused by mutations in the IκB family (23, 30, 31). Other possible ways of NFκB activation are via the TNF receptor associated factor TRAF 1 molecule (32), LMP-1, and CD40 (33, 34). Of interest for this thesis CD30 can also induce NFκB activity (35, 36). The CD30 ligand (CD30L)-CD30 interaction is thought to be of great impor-

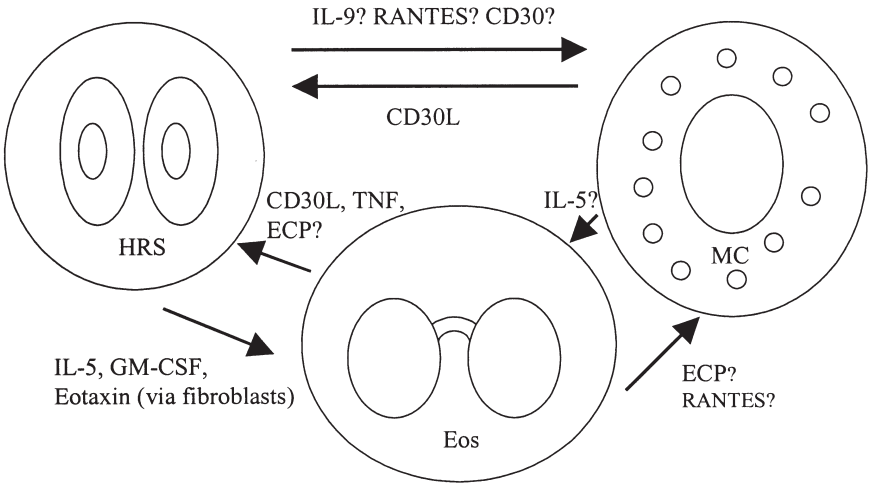

Fig. 1. Interactions between HRS cells, eosinophils, and mast cells.

tance for the HRS cells proliferation (37, 38). CD30 is a member of the tumour necrosis factor (TNF)/nerve growth factor (NGF) receptor superfamily and is expressed on virtually all HRS cells (39, 40). Other possible ways of escaping apoptosis are somatic mutation of the CD95 gene (41), possibly p53 mutations (42), BCL-2 expression (43), or Rb associated mechanisms (44). However, there is no clear evidence for those mechanisms to be relevant in HRS cells escaping apoptosis. A newly described mechanism in escaping Fas-mediated apoptosis is through c-FLIP expression (45).

Several cytokines are produced by HRS cells (46). Among these are interleukin-5 (IL-5), an eosinopoietic factor (47), GM-CSF, also stimulating eosinophils (48), IL-9, an autocrine growth factor for HRS cells (49, 50), possibly involved in mast cell activation (51), IL-13, involved in mast cell activation (52) (Fig 1), IL-6 (53), IL-7 (54), IL-10 (55), and TGF-β (56), a factor considered responsible for the fibrotic bands of NSHL (57).

In addition to the cytokines, several chemokines have been demonstrated to be expressed by HRS cells (46). Examples of these are RANTES, which is a possible factor for mast cell attraction (58–60) and eotaxin (CCL11), which could be an explanation for eosinophil infiltration (59). However, results concerning eotaxin are contradictory and another study showed no expression in HRS cells, but showed expression in fibroblasts and macrophages, secondary to stimulation by tumour necrosis factor (TNF)-α from the HRS cells (61) (Fig 1). Other chemokines found to be expressed by HRS are, for example, thymus and activation related chemokine (TARC) (62), MDC (59), and IP-10 (59).

The HRS cells great capability to communicate with the surrounding cells indicates that the HRS cells and the bystander cells are bilaterally dependent of, and

stimulate the presence of each other. It is therefore valuable to study the bystander cells in order to fully understand the biology of this tumour.

The bystander cells

The surrounding numerous benign cells, previously considered innocent in the disease process, dominate the histologic picture. These cells are for example T- and B-lymphocytes, plasma cells, neutrophils, histiocytes, eosinophils, stromal cells, and, less known, mast cells (63–66). The T-cells in CHL are to a large extent Th2 cells and these are often situated around the HRS cells in a rosette-like manner, possibly recruited by MIG, IP10, and TARC expressed by the HRS cells (62, 66, 67). The bystander cells could be interpreted as an immunological reaction against the tumour. However, it is now known that these bystander cells communicate with the HRS cells via various interleukins and cytokines (46, 68). It is also plausible that the bystander cells, and their communication with the HRS cells, are necessary for the progression of the tumour (38, 46, 47, 68–70). The well-known difficulties in establishing and culturing HRS cell lines are another indicator of the HRS cells' dependence on other factors, such as the bystander cells. Even though the HRS cells are indeed tumour cells, created by a series of events, which maybe initiated by hereditary factors and one or more viruses, the progression of the HL is, after malignant transformation, dependent of a complex web of interactions between cells of immunological and inflammatory importance.

Eosinophilic granulocytes

Eos (Aurora in Latin) is the goddess of the red sky at dawn in Greek mythology (71, 72). Every morning she opens the gates of the sky to her brother Helios (the god of the sun). In histopathological routine staining the acidic dye eosin, named after her is widely used. The eosinophil is characterised by a bi-lobular nucleus and cytoplasmatic granules, stained by the eosin. Being more or less a nymphomaniac, since being cursed by Aphrodite after an unfortunate love affair, Eos is the mother of many children, including the Winds and the stars, among them the Morning Star, Eosphoros (71, 72). The eosinophilic granulocyte is, however, not as quickly passing as the winds, as it remains in tissue for a few days up to weeks (73). Also it is not as abundant in normal tissue as the stars in the sky. However, in many cases of HL eosinophils are present in large numbers (63, 64).

We have previously shown a relationship between cases with abundant eosinophils in the tumour and poor prognosis (64), and this has been confirmed in a large German study (74), but questioned in a recent smaller study (75). There is also a correlation between abundant eosinophils and NS-histology. The correlation to survival is especially pronounced in NS-histology (74). Eosinophilia in the bone marrow of HL patients does not affect prognosis (76), and blood-eosinophilia in HL is associated with a favourable prognosis (77, 78). In other tumours, such as colon cancer (79) and head and neck cancer (80) presence of tumour-associated tissue

eosinophilia is associated with a good prognosis. These data suggest a special role of eosinophils in HL tumours.

The mechanisms behind the presence of abundant eosinophils in HL tumours can be secretion of IL-5 (47) and GM-CSF (48, 81) by the HRS cells (Fig 1). Another possibility is eotaxin, secreted from fibroblasts and macrophages, stimulated by TNF-α from the HRS cells (59, 61).

The reason for the poorer prognosis in eosinophil rich cases of HL has been proposed to be the expression of a ligand to the CD30 receptor (CD30L), which is a member of the TNF/nerve growth factor (NGF) superfamily (38). This interaction can stimulate HRS cell proliferation (38, 68, 82) (Fig 1). As an explanation for the correlation to NS-histology the eosinophils' production of transforming growth factor β (TGFβ) and subsequent stimulation of fibroblasts have been proposed (83, 84). Eosinophil cationic protein (ECP) can also stimulate fibroblasts (85), which could contribute to the NS-histology if high levels of ECP are present in the HL (further discussed below).

In physiological conditions the eosinophils are active in the defence against parasites and viruses, but probably not bacteria (86–88). Other possible roles for the eosinophils are in wound healing and in the defence against carcinomas, via their cytotoxic granule proteins or interaction with other cells in the immune system (79, 80, 89, 90).

The cytoplasmatic granules of the eosinophil contain a number of basic proteins. These proteins are major basic protein (MBP) (91), eosinophil protein X or eosinophil-derived neurotoxin (EPX/EDN) (92), EPO (93), and eosinophil cationic protein (ECP) (94), further discussed below. There are also other proteins present in the granules, such as IL-2 (95), IL-5 (96), catalase (97), IL-6 (98), TNF-α (99), RANTES (100), and bacterial permeability increasing protein (BPI) (101).

There are also other conditions, even more intimately associated with the eosinophils than HL, such as asthma, allergy, atopic dermatitis, and the uncommon condition hypereosinophilic syndrome (73). Possible roles for the eosinophils in these conditions are, for example, tissue destruction and remodelling (102). After completion of their mission the eosinophils are supposed to go into apoptosis (103).

Eosinophil cationic protein (ECP)

ECP was first described in 1977 in Uppsala (94). The protein is a single-chain peptide of 133 amino acids, with a molecular weight ranging from 18–22 kDa depending on level of glycosylation (104). ECP is a ribonuclease (105, 106) and member of the RNase A superfamily (73, 90). In experiments with artificial cell membranes ECP damages these, therefore ECP has been proposed to be a pore-forming protein (107).

The physiological and pathophysiological functions of the protein are not fully understood. ECP has a capacity for parasite killing (87), which is probably one of the functions of ECP in physiologic conditions. It has also anti-bacterial (108), and

anti-viral functions (88). ECP also affects human cells. Of interest for this thesis, histamine release from mast cells is stimulated by ECP (109–111). This is further discussed below. ECP can affect fibroblast function, as it inhibits proteoglycan degradation (85). This mechanism can stimulate fibrosis, possibly, for example, in creating the fibrotic bands in NSHL. ECP also affects coagulation (112), and it affects plasma cells and B-cells, inhibiting Ig production by these cells but not being cytotoxic to them (113, 114).

Mast cells

The name mast cells comes from the German mast zellen, which means 'well fed cells' (115). They were named so by Paul Ehrlich, who also discovered eosinophils, and the name refers to their characteristic cytoplasmatic granules (115, 116). Initially, mast cells were proposed to contribute to the host defence by phagocytosis (117). However well in accordance with their name, this is not their most important role in the body. Instead, the mast cells have important regulatory functions in the inflammatory process (118). They regulate vascular functions (119) and activate other cells (118, 120) involved in this process. The mast cells also have a role in the immune response against parasites and possibly bacteria (121) and in angiogenesis (119, 122). Upon stimulation the mast cells release the contents of their granules, which contain a variety of inflammatory mediators (119). Subsequently, cytokines and other factors released by the mast cells activate different leukocytes (123). The most important factor in differentiation, migration, survival, and growth of mast cells is stem cell factor (SCF) and its receptor Kit (116). On the bad side of the coin, mast cells initiate immediate hypersensitivity reactions, by releasing histamine, stimulated by cross-linked IgE, and, interestingly, also by ECP secreted by eosinophils (90, 109–111). ECP also stimulates the release of tryptase and the synthesis of prostaglandin D_2 (PGD_2), both vasoactive and proinflammatory mediators, in human heart mast cells (110).

Overall, the mast cell derived mediators in inflammation, for example in allergic reactions, can be categorised into the following three groups:

1. Preformed granule mediators: including histamine, heparin, and proteases like tryptase. The proteases are pre-made, stored in granules and released after stimulation of the mast cells. Possible functions of the proteases are tissue remodelling, degradation of microbial structures, and activation of other cells.
2. Lipid-derived mediators: including leukotrienes (e.g. B_4, C_4), and prostaglandins (e.g. D2).
3. Cytokines, such as TNF-α, IL-4, IL-5, IL-6, IL-8, and chemokines (116, 123).

Diseases with known mast cell involvement are asthma, allergy, rheumatoid arthritis, and mastocytosis. Mastocytosis is a rare group of conditions, involving different organs (124). The most common form, affecting the skin, is urticaria pigmentosa, which is indolent. There are also forms with an aggressive course, however these forms are almost always without skin involvement. Symptoms of mastocytosis, mostly related to the histamine release, are, for example, pruritus, skin irrita-

tion, abdominal pain, headache, diarrhoea, and problems caused by abnormal haematopoiesis (124).

Mast cells are also present in different tumours (119, 125), including early reports of their presence in HL (65, 126). The reasons for this accumulation have not been investigated in detail (125). In the tumours the mast cells possibly promote angiogenesis and metastasis (127), and tumour growth (128).

A well-known effect of the histamine release from mast cells is, as mentioned, pruritus, or itching. As will be described in the prognostic factor chapter pruritus is a feature of some cases of HL, and has also been related to prognosis (129, 130). Another uncommon symptom of HL is pain at alcohol intake (131, 132). Interestingly, this alcohol-induced pain can be relieved by intake of anti-histamines, suggesting a role of mast cells also in this unusual symptom (133).

Polymorphisms

A polymorphism is a genetic variant more common in a population than could be explained by a mutation. Polymorphisms that persist over many generations are commonly maintained because no one of the forms gives an overall survival advantage. The occurrence of single-nucleotide polymorphisms differs between different genes, as some contain many and some contain none (134). In a population, a polymorphism is distributed according to the Hardy-Weinberg equilibrium (135). The physiological and pathophysiological role of single-nucleotide polymorphisms is very heterogeneous, as some probably are of no relevance at all, while some alter the function of a protein, or the level of protein production, in such a manner that it can cause disease or affect the course of a disease.

The knowledge of the role of single nucleotide polymorphisms in malignant disease is limited. Polymorphism can never be the single cause of malignant disease, as they are present in a substantial proportion of the normal population. However, there are examples of single-nucleotide polymorphisms correlating with frequency or prognosis of lymphoma (136–138). If the polymorphism is situated in a gene encoding a protein with a pathophysiologic role in a certain disease, and the polymorphism alters the level of production or function of the protein, the polymorphism can, of course, correlate with the presence or progression of the disease.

Epidemiology and aetiology of HL

The age-distribution of HL is characterised by a peculiar bimodal pattern, with a peak at 25–30 years of age and a steadily increasing incidence after 50 years of age (139). It could therefore be speculated that HL in different age groups are different disease entities. There is a difference in histology between the different age groups supporting this idea; NS-histology, the subgroup of special interest concerning eosinophils and mast cells as will be shown in this thesis, is more common in the young adults and MC-histology is more common in the elderly (140, 141). Although the incidence of other lymphomas is alarmingly increasing in the western

world (142) this is not true for HL. The overall incidence of HL has decreased but the incidence in young adults has slightly increased (142–146).

Certain circumstances are associated with a higher incidence of HL in young adults and middle aged: high standard of living in childhood, such as single family housing, small family size, and a high level of maternal education (147). The prevalence of HL is higher in Western Europe and the United States than in Asian countries (140). NS-histology and the first peak of the bimodal age distribution in young adults are features of HL in developed urbanised countries, and MC- and LD-histology are more common in developing countries. In the developing countries the first peak is in young boys (140).

The aetiology of HL is still not elucidated, but there are some clues. At least in young adult patients, an underlying genetic susceptibility is possible, as siblings (148) and, especially, monozygotic twins (149) to HL patients have an increased risk. There are also indications of an underlying infectious agent (150, 151). The only virus that so far has been shown to relate to HL is Epstein-Barr virus (EBV), the virus causing mononucleosis (152, 153). EBV is associated to MC-histology and to higher age, although not all reports are consistent (154–156).

One could speculate on a correlation with asthma and allergy, on the grounds of the presence of eosinophils in the tumours and the correlation to survival (64, 74), however, no clear evidence of such a correlation has been found. Although, in very rare cases HL has presented as an endobronchial lesion (157).

In many cases HL is associated with an immune-deficiency with a reduced amount of CD4+ lymphocytes in peripheral blood (158). Additionally, there have been observations of an immunological impairment in family members of HL patients (159). It has been suggested that a latent membrane protein- (LMP-) 1 immune defect contributes to the development of EBV associated HL (160). Interleukin- (IL-) 10, produced by the HRS cells may also have a role in this, as IL-10 has inhibitory effects on T-cell mediated immunity (161, 162). There are also promising results from treatment of relapsed patients with EBV positive CHL with EBV-specific cytotoxic T-cells (CTL) (163).

Clinical investigations and staging

The staging of lymphomas has proven to be more stable over time than the classification. The different stages are shown in table 2 (164, 165). However, the investigations required for the staging has changed due to advances in radiology, and new knowledge about treatment and prognosis (see below).

The currently recommended investigations are, provided that the HL diagnosis has been confirmed in a biopsy: medical history, physical examination; ear-, nose-, and throat-inspection (biopsies if there are suspect changes); laboratory investigations (B-Hb, differential WBC, trombocytes, ESR, CRP, S-Na, S-K, S-albumin, S-calcium, B-glucose, S-creatinine, S-urate, S-ASAT, S-ALAT, S-bilirubin, S-ALP, S-LDH, optional HIV-test); chest x-ray; computed tomography (CT) (or magnetic resonance tomography (MRT)) scan of chest, axillae, abdomen, pelvis, and for exam-

ple the neck region if it is primarily involved; ultrasonography of the abdomen (two abdominal investigations have been required to rule out HL in the abdomen), CT- or ultrasound-guided biopsies if any suspect finding is seen, and bone marrow biopsy (not necessary in stage I-IIA) (Uppsala/Örebro region Care Programme for Lymphomas, 2001; Treatment of adult patients with early stages of Hodgkin's disease, Nordic Lymphoma Group study, 1999).

^{18}F-fluorodeoxyglucose (FDG) positron emission tomography (PET) is emerging as a very promising investigation to rule out HL, especially in the abdomen (166, 167), for treatment assessment (168), and as a predictor of disease-free survival (169, 170). Previously staging laparotomy with splenectomy was recommended, but this investigation has now been abandoned (20, 171–175). Staging laparotomy with splenectomy is associated with morbidity and the need for this investigation is diminishing since more and more early stage HL patients receive chemotherapy (20, 132). In the past lymphangiography was frequently used as an abdominal investigation but as CT has become available it has also been abandoned (132).

Table 2. Ann Arbor staging (164) of lymphomas, modified in Cotswolds (165).

Stage	
CS	Clinical stage
PS	Pathological stage = staging laparotomy with splenectomy performed (unusual today)
I	One involved lymph node station
II	More than one station, one side of diaphragm, (number of anatomic sites indicated, e.g., II_2)
III	Both sides of diaphragm, if PS further divided into III_1 and III_2:
III_1	With or without involvement of splenic, hilar, coeliac, or portal nodes
III_2	With involvement of paraaortic, iliac, and mesenteric nodes
IV	Disseminated extranodal involvement
Stages further divided into	
A	No B-symptoms
B	B-symptoms (at least one of the following): Unexplained weight loss >10% in 6 months Unexplained fever >38°C Night sweats
E	Involvement of a single extranodal site, or contiguous or proximal to known nodal site
X	Bulky disease: Tumour>10cm or Tumour>1/3 of the thorax diameter at the Th 5-6 level

Prognostic factors

Many prognostic factors have been described for HL. However, when important prognostic factors have been described, then treatment has been adjusted and the patients with negative prognostic signs have been treated more aggressively. Subsequently, these factors have in many cases lost their prognostic impact (176). This has happened for example with bulky disease (177). The Hodgkin-specific and OS have also been improved and are now very good for a malignant disease, which gives few events to study (176). This is all very good for the patient but makes studies on prognostic factors in HL difficult.

In historical descriptions of HL it is evident that the natural history of the disease is highly variable (132). In some cases the disease remains localised for many years but in others it spreads rapidly causing the patient's death after only a short period of time (11, 132). However, untreated the disease is virtually always fatal (11, 132). This huge difference in clinical course necessitated, historically as well as today, reliable prognostic factors (37, 132, 178). These are then used in order to group the patients into risk groups to determine appropriate treatment and to compare treatment results. As the prognosis today is so good, many patients are probably overtreated, with a risk of long-term side effects. This necessitates the finding of factors predicting both good prognosis and poor prognosis (37).

Factors related to the HRS cells, tumour burden, and dissemination of the disease

As early as 1902 it was demonstrated that the spread of the disease correlated to prognosis (11). This has lead to staging systems of which the most important is the Ann Arbor system (164). The correlation between stage and prognosis has been repeatedly confirmed in clinical studies (132, 179, 180); however, nowadays this correlation is diminishing (176). The staging describes foremost the dissemination, but it also gives a hint of the total amount of disease.

As time passed new prognostic factors were described. At first other ways of describing the amount of disease other than dividing it into stage I–IV, were introduced. In Cotswolds in 1988, the Ann Arbor staging system was modified with the tumour bulk (181–184), and the number of sites involved (132, 185) added to the staging (165). In stage II, spread of the disease can vary and the number of involved anatomic sites gives prognostic information (132, 185). Tumour bulk (especially in the mediastinum) has historically proven to be important in early stage HL treated with RT alone (186–189), but in advanced stage, treated with chemotherapy, results are more inconsistent (190–193). Efforts have been made to more accurately estimate the total amount of tumour cells by measuring the total macroscopic tumour burden and combining it with the tumour cell concentration in the HL involved tissue. This approach predicts prognosis (194–196). The relative tumour burden obtained from radiologically measurable tumour burden normalized to body surface area is also promising (197). Soluble CD30 might also correlate with the number of HRS cells and correlates with prognosis (39, 162, 198–200). Different factors reflecting the growth characteristics of the HRS cells are prognostic factors (37).

Expression of Caspase 3 in the HRS cells correlates to favourable prognosis (201). This finding indicates that the apoptosis cascade is important for tumour cell killing by chemotherapy. Serum lactate dehydrogenase, commonly used as a prognostic factor in lymphomas other than HL, also have prognostic significance in HL, probably reflecting tumour cell turn-over (202). Different localizations of the disease can also affect prognosis (203–205).

Factors related to the cellular composition of the tumours

In accordance with the histologic pattern of few tumour cells surrounded by 'bystander' cells, morphological patterns of the tumours have been examined in the search for prognostic factors. Mostly, this is done in the histological classifications described above. The different classifications have given prognostic information (37). Especially the NLPHL subtype behaves biologically in a different manner, giving the division between CHL and NLPHL introduced in the REAL classification (18). However, as a majority of HL patients are in the NS subgroup, an effort has been made to sub-group these (206, 207). Unfortunately the prognostic information yielded could not be confirmed by other groups (208). Among the surrounding cells, activated cytotoxic T-cells have been associated with a negative prognosis in one study (209). Our group has described the negative prognostic impact of tumour eosinophilia (64). This is further discussed in the chapter dealing with the eosinophilic granulocyte.

Factors related to inflammatory activity and the release of cytokines and other factors

Several prognostic factors are related to cytokines and other factors secreted by the HRS cells or the surrounding cells. B-symptoms (37, 132, 210, 211), and high ESR (132, 206, 212), have been introduced as prognostic factors. Similar to ESR, S-Albumin (213), B-Hb (213, 214), WBC (213), and lymphocytopenia (205, 214) are prognostic factors that can be related to cytokines secreted by the HRS or the surrounding cells. In addition to the B-symptoms, pruritus, or itching, has in some reports been demonstrated to have prognostic relevance (129, 130). This is particularly interesting when examining the role of mast cells in HL, as these cells are known to cause itching via histamine release. Soluble VCAM-1 and ICAM-1 have been described as prognostic factors (199, 215). Several cytokines have also been studied as prognostic markers with promising results (37).

Host related prognostic factors

Several other, patient- or host-related, prognostic factors, such as sex (men having a worse prognosis (132, 216)), and old age (132, 216) have been introduced. Patients with immune-deficiency related to HL have a worse prognosis, in a multivariate analysis (217). Several prognostic factors are currently used, as will be described.

Table 3. Risk factors in the international prognostic score (IPS) as described by Hasenclever and Diehl (213).

Parameter	Value
1. Serum Albumin	<40g/L
2. Blood Haemoglobin	<105g/L
3. Sex	Male
4. Age	≥45 years
5. Stage	IV
6. Leukocytosis	WBC ≥15 x 10^9/L
7. Lymphocytopenia	<0.6/L or <8% of WBC

Prognostic scores

The Scotland and Newcastle Lymphoma Group (SNLG) index, valid for all stages, was introduced by Stephen Proctor, Newcastle, in 1991 (214, 216, 218). The patient's age, clinical stage, absolute lymphocyte count, B-Hb, and presence of bulky disease are required for calculation of the index. The index is then calculated by an equation involving these parameters, entered as absolute figures or as different scores. This prognostic index has proven accurate in selecting risk groups (219).

In advanced stage HL an international prognostic score (IPS) has been developed by The International Prognostic Factors Project on Advanced Hodgkin's Disease and described by Hasenclever and Diehl (213, 220). The IPS is used for patients with advanced HL between 15 and 65 years of age and takes into account seven binary adverse prognostic factors (Table 3). It was developed from a database consisting of 5141 patients, of which complete data was available on 1618 patients.

Still there is a need for new prognostic markers, and scores, to be able to minimise treatment in order to diminish side effects in low-risk patients with a long expected remaining life span. These new prognostic markers are likely to be related to the biology of the disease. As the HRS cells are few and surrounded by numerous bystander cells it is logical to search for new prognostic markers in these interactions and the presence and quantity of the bystander cells.

Treatment and prognosis

Treatment of HL is one of the great success stories in non-surgical treatment of malignancies, together with treatment of childhood leukaemias and testicular cancer. Apart from these diseases cure in malignant diseases is usually dependent on radical surgery but in these cases cure is reached through oncological treatment, i.e. medical treatment and radiation, alone. From being a disease that was incurable and virtually always ended with the patient's death, HL has become a highly curable disease (132).

Radiotherapy for treatment of HL was introduced for the first time as early as in 1902 (221). It was subsequently refined (222), and improved the prognosis of HL dramatically (216, 223). When chemotherapy was introduced the treatment was giv-

en as single drugs, rendering only short remissions. A great break-through in HL treatment, and in oncology, was when combined chemotherapy regimens were introduced, initially the MOPP (mechloretamine, vincristine, procarbazine, prednisone) regimen (224–226). The combined chemotherapy led to greatly improved long-term survival.

As treatment today has given an overall favourable prognosis, but with a risk of late side-effects, such as secondary tumours, focus has switched from only achieving a cure, to additionally trying to avoid late side-effects (132, 227–233). This is further discussed in the next chapter. In table 4 a review of chemotherapy regimens used in HL is given.

Treatment in early and intermediate stage (I–IIA) HL is also discussed in the next chapter. Advanced stage HL is generally treated with a full course of chemotherapy, which often means eight courses, followed by RT if the disease was initially bulky or if there is any residual tumour after the chemotherapy (220, 228, 234). With this kind of treatment at least 80–90% of younger patients reach CR. However, the problem is that only 50–70% of advanced stage patients will remain long-term disease-free (203, 226, 235), although recently improved results have been shown with the BEACOPP and escalated BEACOPP regimens (236). Other promising new intensive chemotherapy regimens are Stanford V (237, 238) and PVACE-BOP (219). However, Stanford V was inferior to ABVD, and the MEC combination, in terms of response and FFS in a recent study (239).

Current treatment in advanced stage (IIB–IV) HL in adult patients, 65 years or younger, in the Uppsala/Örebro region in Sweden is: 0–2 risk factors according to IPS: ABVD × 6–8, 3–5 risk factors: standard BEACOPP × 6–8, and 6–7 risk factors: escalated BEACOPP × 6–8. Six cycles of chemotherapy is chosen if the patient is in CR after 2 cycles. In patients above the age of 65 the treatment is CHOP × 6. In all of these groups, chemotherapy is followed by involved field RT, 1.76 Gy per fraction to 30 Gy, if the disease was initially bulky (Care Programme for Lymphomas in the Uppsala/Örebro region, 2001). All patients in advanced stage HL should receive prophylactic antibiotic (trimetoprim+sulfonamid), anti-viral (aciclovir), and anti-fungal therapy (fluconazol). Children (<18 years) are treated according to a separate programme.

Treatment and problems in patients with early and intermediate stage HL

Commonly early stage HL is defined as stage I–IIA, and intermediate stage is defined as early stage with certain risk factors that differ between different groups. Early stage HL has been treated with extended field RT with good results regarding the number of CR and freedom from failure (132, 223, 227, 228). The approach with extended field RT was first introduced by Vera Peters in the 1930–40s and then developed by Henry S Kaplan at Stanford in the 1960s (223). The extended (or locally extended) field in patients with supradiaphragmatic disease is commonly mantle-field, including all supradiaphragmatic lymph nodes commonly involved: i.e. the cervical, supraclavicular, axillary, infraclavicular, superior mediastinal, hilar,

Table 4. Current use and efficacy of chemotherapy regimens used for HL.

Regimen	Introduced (year) and references	1. Current use 2. Efficacy in advanced stage (if evaluated)
MOPP	1964 (224, 225)	1. None 2. Inferior
ABVD (see table 6)	1970s (240)	1. Early/intermediate, some advanced stage HL 2. Standard
MOPP/ABVD	1980s (241)	1. None 2. Equivalent efficacy (inferior, when side-effects considered)
MOPP/ABV	1980s (242, 243)	1. Limited 2. Equivalent (however, see MOPP/ABVD)
ChlVPP	1980s (244)	1. None 2. Inferior
COPP/ABVD	1980s (245)	1. Advanced stage 2. Not compared to ABVD
BEACOPP Baseline	1990s (246)	1. Advanced stage 2. Better
BEACOPP Escalated	1990s (247)	1. Advanced stage 2. Better
EBVP	1980s (248)	1. None 2. Inferior
COPP/ABV/IMEP	1990s (174)	1. --- 2. Equally effective as COPP/ABVD
MVP	(249)	---
VBM	(249)	---
VAPEC-B	1990s (250, 251)	1. --- 2. Inferior
CHOP	...	1. Elderly patients 2. ---
PVACE-BOP	1990s (219)	1. Advanced 2. Unproven
Stanford V	1990s (237, 238)	1. Advanced 2. Inferior
CHOPE	1990s (252)	---

Table 5. Results according to treatment reported by international centres or HL co-operative groups (132, 172, 174, 228, 249, 261-263).

Group + year	Treatment	RFS/FFTF*	OS
EORTC (H1), 1964-1971 (Early/interm.)	RT + Vinblastine vs no chemotherapy	48%	72%
H2, 1972-1976	Staging laparotomy, splenectomy + STNI	76%	79%
	STNI, no laparotomy	68%	77%
H5, 1977-1982	Favourable: Mantle RT	74% (6 years)	96% (6 years)
	STNI	72% (6 years)	89% (6 years)
	Unfavourable: TNI	66% (15 years)	69% (15 years)
	3 MOPP + Mantle RT + 3 MOPP	83% (15 years)	69% (15 years)
H6, 1982-1988	Favourable, negative staging laparotomy: Mantle RT	76% tot H6	88% tot H6
	STNI	---	---
	Unfavourable: 3 MOPP + Mantle RT + 3 MOPP	77% (10 years)	87% (10 years)
	3 ABVD + Mantle RT + 3 ABVD	88% (10 years)	87% (10 years)
H7 (CSIA-IIB, no risk factors), 1988-1993	Favourable: 6 EBVP + IF-RT	92% (6 years)	98% (6 years)
	RT (STNI)	81% (6 years)	96% (6 years)

and supracarinal nodes. In patients with infradiaphragmatic disease the most common extended field is inverted Y. Other extended fields used in many patients are total nodal irradiation (TNI), that is mantle plus inverted Y, and subtotal nodal irradiation (STNI).

Before the development of modern radiology techniques, laparotomy with splenectomy, i.e. PS, was introduced in order to exclude abdominal disease, to minimise treatment in early stage HL (164, 254) (see also Clinical investigations and staging). Unfortunately this procedure has led to an increased incidence of fatal pneumococcal infections and is now abandoned (132, 255).

Early and intermediate stages have also been treated with limited chemotherapy followed by either involved or extended field RT (132, 171–175, 227, 228, 235, 256), with favourable results. For a long time it was debated whether primary com-

Table 6. drugs of the ABVD chemotherapy regimen (see also table 4). A new cycle is started at day 28.

Drug	Dose (mg/m^2)	Day	Group of drug	Major side effects
Adriamycin	25 i.v.	1, 14	Anthracyklin	Haematologic, cardiac
Bleomycin	10 i.m.	1, 14	Other	Pulmonary
Vinblastine	6 i.v.	1, 14	Vinca-alkaloid	Haematologic
Dacarbazine	375 i.v.	1, 14	Alcylating	Haematologic

Table 5, continued

	Very favourable: Mantle RT alone	---	---
	Unfavourable: 6 MOPP/ABV + IF-RT	90% (6 years)	89% (6 years)
	6 EBVP + IF-RT	68% (6 years)	82% (6 years)
GHSG	Intermediate: 2 COPP/ABVD versus 2 COPP/ABV/IMEP + RT	79% (7 years, both arms)	88% (7 years, both arms)
Stanford (CSIA-IIB)	6 VBM + regional RT	88% (5 years)	94% (5 years)
	TNI/STNI	93% (5 years)	98% (5 years)
Stanford S2-S3 (PS IIEA-IIEB), 1974-1980	Unfavourable: STNI/TNI + 6 MOPP	71% (20 years)	71% (20 years)
	Unfavourable: STNI/TNI + 6 PAVe	100% (20 years)	88% (20 years)
Stanford C2-C3 (CSIIA-IIB, bulky, or multiple E), 1980-1990	Unfavourable: 3 PAVe + Mantle RT + 3 PAVe	56% (15 years)	73% (15 years)
	Unfavourable: 3 ABVD + Mantle RT + 3 ABVD	83% (15 years)	100% (15 years)
Stanford-Kaiser Permanente G1 study (CS I-IIA), 1988-1995	2 VBM + Regional RT + 4 VBM	87% (4 years)	
	STNI	92% (4 years)	
Toronto (CSI-II), 1968-1986	...	65% (10 years)	76% (10 years)
Northern Region Lymphoma Group (in Great Britain)** (SNLG index), 1991-1993	Good SNLG index	---	87% (5 years)
	Intermediate SNLG index	---	78% (5 years)
BNLI (CS I-IIA), 1992-1994	2 MVP + IF-RT + 4 MVP	71% (5 years)	97% (5 years)
	2 VBM + IF-RT + 4 VBM	71% (5 years)	93% (5 years)

RFS = Relapse-Free Survival, FFTF = Freedom From Treatment Failure, OS = Overall Survival, EORTC = European Organisation for Research and Treatment of Cancer, GHSG = German Hodgkin's Lymphoma Study Group, BNLI = British National Lymphoma Investigation, RT = Radiotherapy, * Where only treatment failures were given, FFTF was estimated. RFS/FFTF were in some cases estimated from survival curves. ** Population Adjusted Clinical Epidemiology (PACE) study.

bination therapy was beneficial or if those who relapsed after RT could be salvaged with chemotherapy and thus not have a worse prognosis. In a meta-analysis by Specht *et al* (257), this was indeed the case.

Young patients in early and intermediate stages have a good prognosis with a high short-term survival almost regardless of treatment approach. However, there is a risk of serious side effects from the treatment. Most important are the late side effects, for example induced malignancies, such as breast cancer secondary to radiation, acute leukaemias secondary to chemotherapy, lung cancer, and sarcomas (229–233). Hypothyreosis is a very common late side effect and should always be

assessed in HL controls after treatment (231, 232, 258). Patients irradiated to the shoulder region (for example with mantle treatment) often suffer muscular atrophy in that area (231, 232). Heart complications are also of importance, especially in elderly patients (259). Recently fatigue has been emphasised as a complication to HL treatment (260). The late effects are of outmost importance in young patients with a long expected remaining life time. Of the acute side effects the most important are infectious complications (132).

A National Care Programme for HL was introduced in Sweden in 1985 (177). The concept of the programme was to give less intensive treatment than internationally recommended at that time, in order to minimise late side effects. Prognostic subgroups were considered. The aim was to have no more than 20–30% relapses in any group and a high probability of salvaging relapsing patients. Patients with low risk disease were treated with locally extended field RT alone, and patients with high risk disease were treated with a short course (1 cycle of MOPP/ABVD) of chemotherapy, followed by RT. Laparotomy should be performed in some subgroups (see Material and Methods). An early evaluation after a mean follow-up of 5 years for patients diagnosed 1985–1989 (227) showed that the treatment results were favourable and fulfilled the initial objectives of the Care Programme. The model with a short course of chemotherapy followed by RT had been used at certain centres in the early 1980s, and is now introduced more and more into trials and as routine treatment by many large co-operative groups, but the long-term results of large patient groups have not been studied.

An overview of results in early and intermediate stage HL trials is given in table 5. The idea of this table is to fairly comprehensively illustrate the improvement in treatment results over time and to give a background for the results achieved within the Swedish National Care Programme.

Current treatment in the Nordic countries in adult patients is in CHL, stage IA–IIA below 70 years of age with no risk factor 2 ABVD with subsequent 1.76 Gy × 17 to 30 Gy involved field RT, and with risk factor 4 ABVD followed by involved field RT. Risk factors are: ESR B 50, more than two locals, and bulky disease for supradiaphragmal disease. In cases of infradiaphragmal disease the risk factor are: bulky disease, central or pelvis engagement, stage II, and ESR B 50. In patients over 70 years the recommended chemotherapy is CHOP in the Uppsala/Örebro region. NLPHL patients without risk factor are treated with involved field RT to 30 Gy, and in those with risk factors 2–4 ABVD followed by involved field RT to 30 Gy. The drugs and major side effects of the ABVD regimen are listed in table 6.

MATERIAL AND METHODS

Patients and clinical characteristics

A diagnostic tumour biopsy was available from all patients (I–V). Before further analysis all cases were re-evaluated according to the REAL classification (18) (I–IV).

Clinical and pathological staging was made according to the Ann Arbor system (164). Complete remission (CR) was defined as disappearance of all known disease (I–V).

In 54 newly diagnosed patients with HL (mean age 34, male/female ratio 2.4/1) ECP levels in serum (S-ECP) were measured at diagnosis, and a follow-up S-ECP was measured in 17 of these patients (I).

Forty-three HL patients (mean age 33, male/female ratio 1.2/1), and 70 apparently healthy medical students were analysed in the ECP polymorphism study.

Forty-two patients (mean age 35, male/female ratio 1.9/1) were included in the CD30L and mast cell study. In 36 of these patients serum tryptase was measured prior to treatment.

A material of patients diagnosed between 1989 and 1994 has been collected and characterised. In 123 (mean age 43, male/female ratio 1.4/1) of these patients the material was available and sufficient for immunohistochemical analysis and they were included in the mast cell and prognosis study.

Three hundred and eight consecutive patients (median age 31, male/female ratio 1.2/1) in early and intermediate stages, age 17–59 years, were registered in the Swedish care programme (177, 227) in 1985–1992. All of these were included in the study.

Treatment and staging according to the National Care Programme

The staging was made according to the Ann Arbor system (164, 165). However, during the study described in paper V, lymphogram was abandoned as a HL investigation method.

All patients were treated according to the recommendations in the Swedish care programme for HL (177).

In short, the treatment in early and intermediate stages was (227):

In CS+PS IA only radiotherapy was recommended, except in bulky disease, when radiation was preceded by one cycle of MOPP/ABVD. In PS IB one cycle of MOPP/ABVD was followed by mantle irradiation, and in CS IB 3–4 cycles of MOPP/ABVD ± radiation was used.

Patients in CS IIA were treated with 3–4 MOPP/ABVD ± radiotherapy, and in PS IIA only radiotherapy, or, if the disease was bulky, 1 cycle of MOPP/ABVD followed by radiotherapy. In CS IIB recommended treatment was 3–4 MOPP/ABVD ± radiation, and in PS IIB one MOPP/ABVD + radiation.

Patients in stage III, after laparotomy staged as PS III_1A, were treated with radiation, either alone or preceded by one cycle of MOPP/ABVD.

In advanced stages treatment was three to four courses of MOPP/ABVD, followed by RT in cases of initially bulky disease, slow tumour regression or if the patient did not reach CR (234).

In relapses after RT, treatment was chemotherapy if the relapse was within the treated area. If the relapse was outside the treated area and localized new RT could be given. Patients relapsing after chemotherapy were recommended chemotherapy,

in early relapses (within one year) and for most late relapses the MIME (methyl-GAG, iphosphamide, methotrexate, and etoposide)-regimen was used, with or without subsequent high-dose therapy with stem cell support (264).

In recent versions of the Care Programme treatment recommendations are as described in the Treatment chapters in the background section. Children were treated according to separate protocols.

Serum proteins

S-ECP was measured by means of a commercially available kit (Pharmacia and Upjohn Diagnostics, Uppsala, Sweden), immediately after the sampling (I). The 95% range of S-ECP in healthy individuals is 4–16 μg/l (265). Serum levels of tryptase, a mast cell specific proteinase, were also measured using a commercially available kit (Pharmacia and Upjohn Diagnostics, Uppsala, Sweden).

Immunohistochemistry

The tissue specimens were routinely fixed in neutral buffered formalin, embedded in paraffin and sectioned in 3 μm thick sections. Prior to the staining the slides were deparaffinised.

The eosinophil-specific monoclonal antibodies (mAb:s) EG 1 and EG 2 (Pharmacia-Upjohn Diagnostics, Sweden) (266) were visualised with the APAAP technique.

In order to visualise expression of CD30L in mast cells in HL tumours, a double-staining technique for CD30L and the mast cell specific protease tryptase was developed. The anti-CD30L mAb (IgG2b isotype, Genzyme Diagnostics, Cambridge, MA) was visualised with the DAB method in a Ventana machine (Ventana Bio Tek systems, Tucson, AZ, USA) according to the manufacturer's instructions. After this procedure the slides were washed and microwave-treated, before they were stained with an anti-tryptase polyclonal rabbit antibody (pAb) (267) utilising the APAAP method. The tryptase-specific mAb provided by Dr. L.B. Schwartz, MCV, Richmond, VA (268) was visualised with ABC/HRP and DAB technique.

The positive and double-positive cells were counted in 10 randomly selected high power fields (HPF, 500x). An ocular with a lattice square net was used. In NS cases only cellular areas were counted.

Cell cultures

One human mast cell line, HMC-1 (269), and one human basophilic cell line, KU 812 (270) were used. Culturing was as previously described (271, 272). Human mast cells were obtained by culturing umbilical cord blood cells in complete Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 50 ng/ml stem cell factor (Immunex, Seattle, WA, USA), and 10ng/ml IL-6 (Peprotech, London, UK) (273, 274). Mast cell numbers and purity were determined by staining for tryptase. The purity of mast cells was >92%. U-698 was used as a positive control for CD30L (275). U-2932, a non-Hodgkin lymphoma derived cell line, found to be CD30-negative (unpublished

observations) was also used. The HL-derived cell lines used were HDLM-2 (276), L 540 (277), KM-H2 (278), and DEV (279). Other cell lines used were Cl.MC/C57.1, a cloned growth factor-independent mouse mast cell line of BALB/c origin (280), MCP5/1, a growth factor-dependent mouse mast cell line (281), and P815, a growth factor-independent mouse mastocytoma cell line (282).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was prepared using TriPure isolation reagent (Boehringer Mannheim, Mannheim, Germany). RNA from HMC-1 cells was treated with Heparinase I (Sigma). One microgram of RNA was reverse-transcribed by oligo-p (dT)15 priming using AMV reverse transcriptase (kit from Boehringer Mannheim). For human CD30L amplification, a 571 bp fragment starting from position 312, and for mouse CD30L amplification a 554 bp fragment from position 242 were generated. The PCR products were size fractionated on agarose gel, stained and photographed. The identity of the products was confirmed by restriction enzyme cleavage and sequencing.

Flow cytometry

The cells were stained with a mAb against CD30L (M81, Immunex), Kit (YB5.B8, Dr Ashman, Adelaide, Australia), or isotype controls. Following incubation and washing they were incubated with a FITC-labelled F(ab´)2 fragment of rabbit-anti mouse Ig (DAKO, Glostrup, Denmark). The cells were then analysed using a FACScan (Becton Dickinson, Mountain View, CA, USA).

Endonuclease restriction digestion

The samples were amplified with primers producing a 644 bp fragment. Seventeen microliters of the PCR product (non-biotinylated) was incubated with 10U *Pst* I in an appropriate digestion buffer (Invitrogen). The samples were digested over night and subsequently analysed on an 1.5 % agarose gel containing ethidium bromide. To verify that the highly homologous DNA region of the EPX/EDN (*RNASE*2) gene had not been co-amplified, the 644 bp PCR fragment was subjected to the ECP gene specific *Cla* I endonuclease digestion (283). This resulted in complete digestion (and two bands of sizes 241 and 403) showing that only the region containing the ECP gene had been amplified (data not shown).

Calculation of isoelectric point (pI)

The pI's of the ECP-variant proteins were calculated by VectorNTI software (Infomax, North Bethesda, MD).

Statistical analysis

All statistical analyses were made utilising the Statistica software (version 4.5–6.0 StatSoft, Tulsa, USA). HL-specific survival, OS, and RFS were visualised with Kaplan-Meier graphs and compared with the log-rank significance test. OS was

Table 7. S-ECP levels according to clinicopathologic characteristics in 54 HL patients.

Patient characteristics:	Number of patients:	S-ECP Mean±SD:	P-value:
Total	54	25.4±17.4	
Sex			
Female	16	27.8±20.4	
Male	38	24.4±16.2	0.6
Stage			
I+II A	36	22.2±14.7	
IIB-IV	18	31.8±20.9	0.1
Symptoms			
A	38	23.5±15.6	
B	16	30.1±20.8	0.3
Histology			
Non-NS	25	21.7±17.9	
NS	29	28.7±16.6	<0.05
MC	18	22.0±16.3	
NS	29	28.7±16.6	0.1
Bulky disease			
Non bulky	40	22.7±15.0	
Bulky	14	33.4±21.6	0.06

defined as time from diagnosis to death of any cause. HL-specific survival was defined as time from diagnosis to death with HL. RFS was defined as time from diagnosis to first relapse. Patients who never reached CR (or CRu) were not censored and their time of follow-up was zero in this analysis. Cox hazards regression model was used to compare the importance of different prognostic variables.

Differences in proportions were evaluated with the Chi^2-test, or Fisher Exact test, if the frequency in any group was too small (<5). To compare the actual distribution of a polymorphism with the calculated Hardy-Weinberg equilibrium distribution the Chi^2 test was used. The Mann-Whitney U Test was used as a non-parametric test. To investigate correlations between two continuous parameters the Spearman test was

Table 8. distribution of the 434 genotype according to histology in 43 HL patients.

Histology:	Number of patients:	Number of 434GG	Number of 434GC	Number of 434CC	p-value
NS Histology	25	18	6	1	0.03
All Non-NS	18	7	10	1	
MC	15	6	8	1	
LD	0	0	0	0	
LPn	3	1	2	0	

used. We also used an analysis of variance (ANOVA), followed by multiple comparison using Fisher's method.

RESULTS

Eosinophils in HL

Tissue eosinophilia and serum ECP

S-ECP was measured in 54 newly diagnosed HL patients. Mean S-ECP was 25.4 μg/l (range 2.2–71.7 μg/l). In 61% of the patients S-ECP was higher than the upper normal limit (16μg/l). The S-ECP correlated to the number of eosinophils in the tumours ($p=0.01$) and to NS histology ($p<0.05$). There was also a correlation between S-ECP and the negative prognostic factor ESR ($p<0.01$), and WBC ($p=0.0002$), and there was a tendency of an association between S-ECP and bulky disease ($p=0.06$). The number of eosinophils stained with EG 2 correlated to high ESR ($p<0.05$), and to high leukocyte count ($p=0.02$). Follow-up values of S-ECP were, in most of the cases where it was measured, lower than S-ECP at diagnosis. S-ECP levels according to clinicopathologic characteristics are shown in table 7.

ECP polymorphisms

As we had shown a correlation between eosinophils, NS-histology, and survival (64), and a correlation between S-ECP, eosinophils in the tumours, NS-histology, and negative prognostic factors, it was logical to study if polymorphisms, possibly affecting the function of ECP, could affect the course of HL.

Single nucleotide polymorphisms in the gene coding for ECP were found at three different positions; 277(C>T), 434(G>C), and 562(G>C), base numbers referring to gene bank no. NM_002935. Polymorphisms at position 277(C>T) and 434(G>C) result in the following amino acid shifts: arg45cys and arg97thr. The polymorphisms 277(C>T) and 562(G>C) were present only in a heterozygous form and 434(G>C) was present in both a heterozygous and a monozygous form. pI calculations of the variant proteins showed that the pI was 10.2 for arg45cys and 10.5 for arg97thr. The original ECP has a pI of 10.7.

As 434(G>C) was located at a restriction endonuclease site for the enzyme *Pst* I, inhibiting the DNA cleaving activity of the enzyme, the material screened by sequencing could be RFLP analysed by *Pst* I digestion. The 434GG form gave complete cleavage, the 434CC did not cleave at all, and the heterozygous form, 434GC, gave both cleaved and uncleaved fragments. The results of this analysis in the seventy apparently healthy subjects studied were in accordance with the sequencing results.

Of the seventy healthy subjects, 3 (4%) were found to be homozygous for the 434C genotype and 30 (43%) were heterozygous, 434GC. The 277(C>T) polymorphism was found in a heterozygous form in 2 (3%) of the 70 subjects. The frequencies of alleles for both polymorphisms were in Hardy-Weinberg equilibrium.

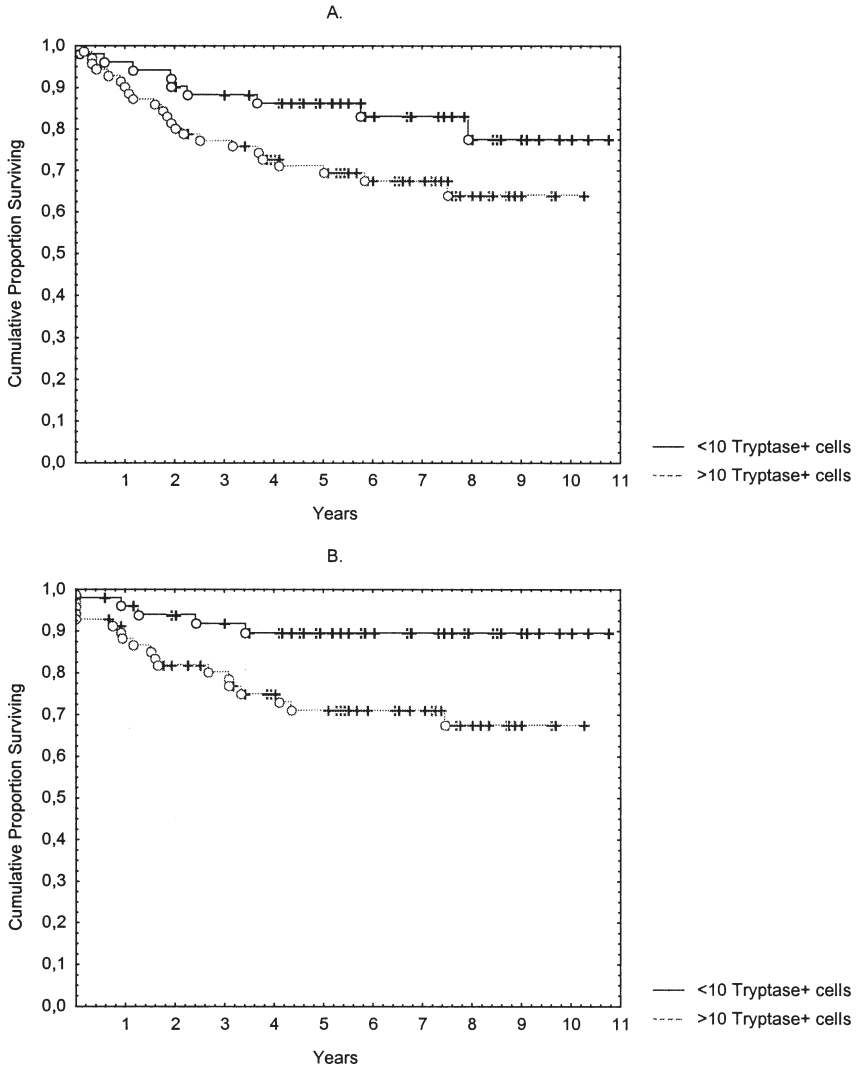

Fig. 4. A. Overall survival according to number of mast cells (p=0.06, n=123). B. Disease-free survival according to number of mast cells (p<0.01, n=123).

The prevalence of the 434(G>C) polymorphism was investigated in 43 patients with HL. The results were similar to those of the healthy subjects, with 25 (58%) 434GG, 16 (37%) 434GC, and 2 (5%) 434CC. The 434GG genotype, however, was over-represented in patients with NS histology (72% versus 39% in non-NS patients, p = 0.03), which is seen in Table 8. Furthermore, the negative prognostic factor ESR was higher among patients with the 434GG genotype (p = 0.009). B-Hb tended to be lower for the 434GG genotype (p=0.06).

Table 9. Laboratory parameters according to number of mast cells in the tumours of HL patients.

Laboratory parameter	<10 MC/10HPF	>10 MC/10HPF	P-value
Mean WBC	$7.3\pm0.4x10^9$	$9.5\pm0.5x10^9$	0.002
Mean B-Hb, g/l	132.4±2.8	125.3±2.1	0.03
Mean ESR	37.3±5.0	43.0±4.9	0.4

MC=mast cells, HPF=high power vision field, WBC=white blood cell count, B-Hb= Blood haemoglobin, ESR=erythrocyte sedimentation rate.

Mast cells in HL

Expression of CD30L on mast cells and stimulation of HRS

Considering CD30L had been discussed as a mechanism of eosinophils stimulating the HRS cells (38), and that we noted that cells stained for CD30L in HL morphologically resembled mast cells, we decided to explore this further. One way of doing this was to develop a double staining technique for tryptase, detecting mast cells, and CD30L.

Tryptase-positive cells were visualised in all 42 examined tumours. Fifty percent of these expressed CD30L and 66% of the CD30L-positive cells were tryptase-positive cells. Double-positive cells were found in 95% of the tumours. More tryptase-positive and CD30L-positive cells were found in NS histology compared to the other histological groups, although the differences were not significant. No double positive cells were found in LD histology. There was a tendency to an association between double-stained cell infiltration and bulky disease (p=0.08). The tryptase levels in serum were within the normal range (284) (mean 5.1 ng/ml), and did not correlate to the number of mast cells in the tumours.

Expression of CD30L mRNA was detected using RT-PCR in the human mast cell line HMC-1 and in two preparations of stem cell factor dependent cord blood cultured human mast cells. CD30L mRNA was also found in the murine mast cell lines C57 and P815 and in murine bone marrow derived cultured mast cells, but not in the murine mast cell line MCP5/1, although expression could be induced by activation with ionomycin. Expression of CD30L on the surface of HMC-1 cells and *in vitro* developed human mast cells was measured by flow cytometry. Almost all HMC-1 cells were strongly positive for CD30L and CD30L was also shown on the *in vitro* developed human mast cells.

To determine if mast cells can stimulate proliferation of HRS cells via CD30L-CD30 interaction co-culture assays were performed. The CD30L positive mast cells induced a dose-dependent increase in [^{3}H]-Tdr uptake in the HL cell lines HDLM-2, DEV, KM-H2, and L-540. The increase in [^{3}H]-Tdr uptake that was seen in HRS cell lines could be inhibited by anti CD30L mAb. The spontaneous uptake of [^{3}H]-Tdr in HDLM-2 was also inhibited by this antibody. Isotype control antibodies did not affect the proliferative response. The CD30L negative cell line KU812 did not

increase the uptake when co-cultured with HDLM-2 cells. Instead, a reduction in proliferation was seen. HMC-1 cells additionally induced an increased [^{3}H]-Tdr uptake in the Non-Hodgkin lymphoma cell line U-2932.

Mast cell infiltration and prognosis

Tryptase positive mast cells were immunohistochemically visualised in 113 (92%) of the tumours and in every histopathological subgroup. The mean number of mast cells in the tumours was 19.2 (range 0–101) mast cells/10 HPF. Seventy-one (58%) tumours had >10 mast cells/10 HPF and 52 (42%) had <10. Mast cells were located both in proximity to the HRS cells and in areas of fibrosis. The mast cell infiltration correlated to NS-histology (p=0.008) and there was a tendency to an association between number of tumour mast cells and stage (p=0.07). RFS and OS according to number of mast cells in the tumours are shown in Figure 4. WBC and B-Hb correlated to number of tumour infiltrating mast cells, as shown in Table 9. ESR also tended to correlate to number of mast cells in the tumours.

HL in early and intermediate stages – treatment and prognosis

Overall 304 (99%) of the patients reached CR (or CRu) after treatment. RFS at five years was 80% and at ten years 74% for all patients. In the individual stages the RFS was 79%–95% except in PSIII1A where it was 53%. HL-specific five-year survival was 97% and HL-specific ten-year survival 92% (Fig 5). OS was 93% at five years and 85% at ten years. In total, 74 (24%) patients relapsed a first time, and 20 (6%) a second time, and 18 (6%) died in their disease.

RFS and OS were significantly worse in patients treated with radiotherapy alone compared to those treated with a short course of chemotherapy followed by RT in the whole material (p=0.0003 and p=0.004 respectively). Also when radiotherapy alone was compared to all those treated with chemotherapy (including full chemotherapy) the differences in RFS and OS were significant (p=0.006 and p=0.04 respectively). The difference in RFS was also pronounced in patients with bulky disease (p=0.0005). Patients treated with one course of MOPP/ABVD and radiotherapy had an excellent outcome with, at ten years, RFS 95%, HL-specific survival 98%, and OS 98%.

Concerning prognostic factors, in univariate analyses, histology, age, ESR, and stage significantly affected the RFS, but not sex, B-Hb, or bulky disease. In a multivariate analysis, only stage and MC-histology significantly affected the RFS. If treatment (RT versus chemotherapy±RT) was included in the analysis, treatment, stage, and ESR significantly affected the outcome. RFS was not significantly worse in patients with 3 risk factors or more according to the international prognostic score (IPS).

Of the 44 (14%) patients that died, 18 (6%) died from HL. Of the 26 patients that died without HL, the cause of death was in eight cases other malignancies, six serious infections, three myocardial infarctions, one pulmonary embolism, one cerebrovascular lesion, one retroperitoneal bleeding, and one suicide. In four cases the

cause of death was missing, and in one case probably without HL, but with no autopsy performed.

DISCUSSION

Bystander cells in HL

The rationale for studying the surrounding cells in HL is quite clear; if the tumour cells do not survive without this inflammatory infiltrate, knowledge about the surrounding cells and their interactions with the HRS cells can give both new biological prognostic factors and possible therapeutic approaches, possibly less toxic than the traditional RT and chemotherapy. The results presented here show that both eosinophils and mast cells have a role in the pathogenesis of HL, and that there are similarities in their biological functions in HL. They should thus be considered not innocent bystander cells but guilty opportunists.

Eosinophils in HL

Tissue eosinophilia

In tumours other than HL abundance of tissue eosinophils is associated with a better prognosis, assumed to be due to the cytotoxic activity of the eosinophils (79, 80, 285, 286). However, in HL heavy eosinophilia in the tumours is associated with a worse prognosis (64, 74), although this is debated (75, 208, 287, 288). Furthermore, HL patients with blood eosinophilia have a better prognosis (77, 78), and patients with bone marrow eosinophilia have a similar prognosis to those without (76). This suggests a profoundly different role of the eosinophils in HL compared to other tumours. A possible explanation of the worse prognosis in patients with abundant eosinophils in their tumours could be that the eosinophils stimulate the proliferation of the HRS via CD30L-CD30 interaction (38, 69, 82, 289). However, we show that the predominant CD30L expressing cells in HL tumours are the mast cells.

In paper I there is a tendency that tumours with NS-histology contain more eosinophils. This finding is demonstrated in previous studies (63, 64, 75, 290). The association between the eosinophils and the sclerosis associated with NS histology is not completely elucidated but a number of theories to explain it exist. One possibly contributing factor could be the secretion of TGFβ from the eosinophils (83, 84). Heavy molecular weight TGFβ levels in urine are elevated in NSHL patients (83), and much of the TGFβ is secreted from eosinophils (84). Another possible mechanism is the ability of ECP to inhibit proteoglycan degradation in fibroblasts (85). Interestingly, prognosis in patients with NS-histology in general is relatively good (287), but patients with NS-histology and eosinophilia in the tumours have a poor prognosis (64, 74).

The eosinophil infiltration, as estimated by EG2 positive cell count also correlates with WBC and there is a strong tendency to an association with ESR. This suggests a role for the tumour eosinophils in the inflammatory activity of HL. This is further discussed below.

ECP

The elevated S-ECP levels in a majority of HL patients and the strong correlation between S-ECP and eosinophil infiltration in the tumours shown in paper I implicate that large amounts of S-ECP is produced by eosinophils in the tumours. It also indicates that the ECP produced by these eosinophils has a role in the disease process. This is supported by the follow-up measurements, although analysed in relatively few patients, where the S-ECP in most cases had diminished as the patients went into remission. S-ECP could thus be used to follow treatment effects, especially for possible treatments directed against the eosinophils.

The idea of ECP being important in the disease process is supported by the correlation of ECP and histology. One possible role of the ECP is in the fibrosis, as both S-ECP and tumour eosinophilia are associated with NS-histology. A possible mechanism of enhancement of the fibroblasts function is that ECP inhibits proteoglycan degradation in fibroblasts (85).

In paper I, S-ECP tended to correlate to bulky disease. Nearly all patients with bulky disease had NS histology. Hence, a relationship between many eosinophils, being able to stimulate fibrosis, NS-histology, and large tumours is seen. Therefore, it could be speculated that large tumour bulk is caused by eosinophil-driven fibrosis and CD30L-CD30 stimulation of the HRS cells. However, the association between S-ECP and bulky disease could also partly be explained by a proportional relationship between the tumour volume and S-ECP. In previous studies no correlation was seen between eosinophil infiltration and bulky disease (64, 74).

The correlations between ECP, especially 434GG ECP (below), and disease activity could hypothetically be explained by an activation of the mast cells or the mast cells expression of CD30L by ECP, secreted from the eosinophils in the tumours. There are earlier studies suggesting a stimulatory effect of ECP on mast cells (109–111). This could also possibly explain why the eosinophils and ECP have a disease-supportive role, so distinctly different from the role in other tumours, where the eosinophils appear to be a defence against the tumour, and the ECP to have mainly a cytotoxic effect against the tumour cells (79, 80, 285, 286). These results also support the idea that ECP is not cytotoxic against the HRS cells. This is also in line with results showing absence of cytotoxic activity of ECP against B-lymphocytes but inhibition of immunoglobulin production in these cells (113, 114), as HRS cells are B-cell derived and lack Ig expression (21–23).

The correlation of S-ECP and WBC adds support to the theory of eosinophils and ECP as part in the disease process. Secreted growth factors from the tumour eosinophils or HRS could stimulate the bone marrow resulting in a high WBC.

The lack of significant correlations between S-ECP levels and survival in this study was probably due to short follow-up and a favourable prognosis.

ECP polymorphisms

We describe three different polymorphisms, two of which give amino acid substitutions in the ECP protein. The 434(G>C) changes an arginine to a threonine at posi-

tion 97 (arg97thr). The 434(G>C) polymorphism is common, as almost half the population has this substitution either as a heterozygous or homozygous variant. The frequency of 434(G>C) that we found (26%) is almost the same as the frequency shown by Zhang and Rosenberg (28%) (291). The polymorphism is in Hardy-Weinberg equilibrium, which means that it is randomly distributed in the population.

Considering the polymorphism is equally distributed in HL as in the normal population the polymorphism does not seem to have any aetiological role in HL. However, it seems to have a role in the pathophysiology of the disease. The finding of a correlation with NS-histology suggests an altered biological function of the protein affecting the development of this specific histologic type. This could hypothetically be explained by an impaired ability of the ECP to affect fibroblasts via inhibition of proteoglycan degradation (85). The correlation to high ESR and the tendency to lower B-Hb in 434GG patients also indicates a link to the inflammatory process. A correlation between the 434GG genotype and allergic symptoms supports an altered biological function of the ECP depending on genotype (292). The influence on inflammation and on the course of the disease in HL could also be explained by a change in the ability to stimulate mast cells, which we demonstrate have a role in HL. The different forms of ECP could, for example, hypothetically differ in the ability to stimulate CD30L expression in mast cells. The stimulation of mast cells by ECP has been studied (109–111), but not a possible stimulatory effect on CD30L expression.

Mast cells in HL

Expression of CD30L and stimulation of HRS

We also show that mast cells express CD30L, that they are the predominant CD30L expressing cells in HL, and that they can stimulate HRS cells via CD30L-CD30 interaction.

Mast cells were visualised in all examined HL tumours. The presence of mast cells in HL tumours has been demonstrated, but the role of these cells had not been examined before this study (65, 126). Approximately half of the mast cells expressed CD30L. Previously, CD30L has been shown on a number of other cell types, for example subsets of activated T cells, neutrophils, histiocytes/macrophages, and eosinophils (38, 68, 293), but not on mast cells. In this material mast cells were also the dominating cell type expressing CD30L, showing that the CD30L expression of mast cells is more prevalent than that of eosinophils. We also confirmed the mast cells expression of CD30L by demonstrating that a human mast cell line and in vitro developed human mast cells expressed CD30L mRNA and protein. Interestingly, mast cells in LDHL did not appear to express CD30L, therefore it can be speculated that this expression is regulated by, for example, T-lymphocyte derived cytokines. However, only two LD cases were examined.

When co-cultured with HRS cell lines mast cells were shown to stimulate growth

of the HRS cell lines, demonstrated as a dose-dependent increase in [^{3}H]-Tdr uptake, and this was abolished by addition of anti-CD30L mAb. Stimulation of HRS cell lines via recombinant CD30L and CD30L expressed by eosinophils have previously been shown (38, 82). This ability to stimulate the HRS cells via CD30L-CD30 interaction taken together with the fact that most CD30L expressing cells in the tumours are mast cells suggest an important role for the mast cells stimulating the tumour cells in HL after being attracted, activated or stimulated to proliferation by the HRS cells. The attraction of mast cells can be via RANTES (60) and possibly the activation via IL-9, as IL-9 has been shown to be expressed by HRS cells (50) and it has also been shown to stimulate murine mast cells. However, stimulation of human mast cells via IL-9 has not been demonstrated. Preliminary results indicate an up-regulation of the mast cells CD30L expression upon IL-9 stimulation (not published). Probably the HRS cells and the mast cells can also interact via, for example, IL-13 (294). We also showed an inhibition of the spontaneous [^{3}H]-Tdr uptake in an HRS cell line by the anti-CD30L mAb. This suggests an expression of CD30L on the HRS creating an autocrine loop. We have also detected CD30L mRNA and protein in HRS cell lines in preliminary experiments, supporting this theory (not published).

Mast cells and prognosis

Our previous findings led to a study of the clinical role and possible relationship to prognosis of mast cells in HL. It is demonstrated that number of mast cells in the tumour predicts a worse relapse-free survival in HL patients, and that higher number of mast cells correlates with NS histology.

Mast cells were detected in most of the cases examined and in every histopathological subgroup. A cut-off point of 10 mast cells/10 HPF was chosen because of the distribution of mast cell numbers seen in this material and the material in which we studied CD30L.

The disease-free survival was significantly poorer in patients with >10 mast cells/10 HPF and the OS tended to be worse than in those with <10 mast cells/10 HPF. Further attempts of sub-grouping those with >10 mast cells/10 HPF did not yield any further information. In stage IIB–IV and bulky disease both disease-free survival and OS tended to be worse in patients >10 mast cells/10 HPF. Hence, a stimulatory effect of the presence of mast cells, not accentuated by an abundance of these cells is implicated.

NS histology correlated to abundant mast cells in the tumours. The number of mast cells also correlated to known prognostic factors, such as high WBC, low B-Hb. This indicates a role of the mast cells both in the inflammatory reaction of HL and in the progression of the NS-histology.

The number of mast cells correlates thus to unfavourable prognosis and might be an explanation to the previously mentioned correlation of itching and bad prognosis (129, 130). These results, taken together with results in paper I–III demonstrate that

the eosinophils and the mast cells are not innocent bystander cells but guilty opportunists.

Early and intermediate stages

Treatment and prognosis

Generally favourable treatment results in patients with early and intermediate stage HL, treated with limited tailored treatment, in an unselected population-based material is shown. The overall HL-specific survival was 92% and OS 85% after 10 years. The initial aim of at most 20–30% relapses was obtained in all subgroups except PSIII1A, and the aim of salvaging most relapsing patients was also achieved.

The treatment recommended in the Swedish National Care Programme was, when it was introduced, in many cases less extensive than internationally recommended. In a comparison with the work of international groups during the same time our results are comparable (Table 5). From this comparison we conclude that relatively limited tailored treatment can be given in order to diminish the late side effects without compromising the outcome of the patients.

The best results in this material were obtained for patients receiving limited chemotherapy followed by RT. Patients with risk factors (bulky disease or B-symptoms) receiving combined therapy had an even better survival than those without risk factors receiving RT alone. In patients with bulky disease treated with RT alone, violating the principles, results were much worse than in those receiving combined therapy. This is in line with the present international trend to treat all patients with early or intermediate stage HL with limited chemotherapy followed by IF-RT (171–175, 295). The concept is that addition of a short course of chemotherapy allows limitation of the RT volumes, in order to potentially diminish long-term side effects. However, these principles were introduced prior to this trend. In this material the given RT was extended (mantle) field RT, therefore the results are not directly comparable to results from limited chemotherapy and IF-RT. However, several studies have shown that IF-RT is as effective as extended field RT when it is preceded by a short course of chemotherapy (296–298).

Another international trend some years ago was the abolishment of staging laparotomy with splenectomy (171–175). Our results indicate that laparotomy can be avoided since the recurrence rates were comparable in CSIIA and PSIIA, treated with RT (table 2, paper V).

The poor prognosis in patients over the age of 40 is previously described (299, 300). As these patients are not within the group of elderly patients, over 60 years, with established poorer prognosis (228, 301), a possible conclusion could be that patients within different age segments of the population under 60 years could gain from separate treatment strategies. However, it is not possible to draw such a conclusion on these results alone. A possible explanation of the different outcome could be a difference in histopathology, with more MC patients in this group. Also, there was no impact of age in the multivariate analysis, in contrast to the impact of MC-

histology. However, it was not possible to histopathologically re-evaluate these cases and it is possible that some of these cases would be classified as lymphomas other than HL today.

Since these patients were treated changes have been made in the Swedish recommendations. Staging laparotomy with splenectomy has been abandoned. Extended field (mantle and (S)TNI) RT and RT alone is no longer recommended (except IF-RT in LP-histology, stage I+IIA without risk-factors). The recommended chemotherapy was in 1994 changed from MOPP/ABVD to MOPP/ABV, and in the Nordic trial from 1999 to ABVD.

There are several ongoing international trials on early and intermediate stages. On the basis of previous EORTC studies (Table 5) the current H9 trial has been designed, in co-operation with GELA (228). Standard-treatment in the favourable group is 6 EBVP followed by involved field radiotherapy (IF-RT) (36 Gy), and this is compared to 6 EBVP + IF-RT (20 Gy) or no radiotherapy at all. The unfavourable group of patients is randomised between 6 ABVD, 4 ABVD or 4 baseline BEACOPP, all followed by IF-RT.

Josting and Diehl (172) conclude that recent trials have reported excellent results with combined-modality treatment in favourable (early stage) HL and that in early stage unfavourable (intermediate stage) new chemotherapy regimens can decrease failures and still reduce the need for radiation. In this paper they describe ongoing GHSG trials for these two groups comparing 2–4 ABVD + 20–30 Gy involved field radiotherapy for favourable (early-stage) and 4 ABVD versus 4 BEACOPP + 20–30 Gy involved field radiotherapy for unfavourable (intermediate stage).

The aim of both minimising therapy, by for example substituting extended field RT with limited chemotherapy and IF-RT, and investigations, by abandoning laparotomy with splenectomy, is to minimise the late side effects in these patients with a long expected remaining life span. These results from a population-based material with relatively long follow-up can give an indication about the long-term results of such an approach.

The relatively limited tailored treatment in the Swedish Care Programme thus fulfilled its purpose of producing favourable results, and additionally in an international perspective. Patients with risk factors treated with a short course of chemotherapy followed by RT had an excellent outcome. Most patients relapsing could be salvaged with chemotherapy. The late toxicity was also limited, further strengthening this approach. However, it is still too early to reliably evaluate the risks of secondary malignancies and cardiac mortality.

Prognostic factors

In paper V, the risk factors that remained after univariate and multivariate analyses were: stage ($PSIII_1A$ versus the others), ESR, and MC histology. Treatment also affected the outcome if it was included in the analysis, further emphasising the advantage of combined therapy. The IPS, initially developed for advanced stages, did not add any prognostic information in this material. The SNLG index was not

evaluated in this material, but can be considered in future Swedish or Nordic trials.

The risk factors described in the current care programme are, for patients with supradiaphragmal presentation, bulky disease, number of involved sites, and ESR.

As there is a need for identifying patients where treatment can be further minimised to avoid late side effects, further research is necessary concerning prognostic factors, especially as the IPS did not add prognostic information in this group. These prognostic factors are possibly found in the biology of the disease, i.e. in the role of the tumour cells (194), the bystander cells, and their communication with each other (64, 74).

CONCLUSIONS

HL patients express high levels of S-ECP, probably originating from eosinophils infiltrating the tumours. High S-ECP correlated to negative prognostic factors and NS-histology.

The 434(G>C) polymorphism in the ECP gene is common in the normal population. In HL it correlates to NS-histology and to high ESR.

Mast cells express CD30L *in vitro* and *in vivo*, they are the predominant CD30L expressing cells in HL tumours, and they can stimulate HRS cells *in vitro* via CD30L-CD30 interaction.

Abundant mast cells in the tumours predict a worse relapse-free survival in HL patients, and a high number of mast cells correlates with NS histology.

Generally treatment results were favourable in patients with early and intermediate stage HL, treated with limited tailored treatment. Risk factors that remained after univariate and multivariate analyses were: stage, ESR, and MC-histology. Treatment also affected the outcome if it was included. IPS could not be used to predict outcome, therefore new prognostic factors would be valuable.

These results suggest that the mast cells and eosinophils in HL should not be considered innocent bystanders but guilty opportunists. New prognostic factors are needed in early and intermediate stage HL. Knowledge about the interactions between these guilty opportunists and the HRS cells could provide such factors.

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