From the Institute for the Health of Women and Children / Department of Pediatrics
The Sahlgrenska Academy at Göteborg University, Göteborg, Sweden

IMMUNE RECONSTITUTION AFTER CHILDHOOD LEUKEMIA

Aspects on immunizations and effects of Ara-C on the innate immune system

Torben Ek

GÖTEBORG
UNIVERSITY

2005

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TORBEN EK
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Karolinska sjukhuset, Stockholm

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I. Ek T, Mellander L, Hahn-Zoric M, Abrahamsson J. Intensive Treatment for Childhood Acute Lymphoblastic Leukemia Reduces Immune Responses to Diphtheria, Tetanus, and Haemophilus influenzae Type b. J Pediatr Hematol Oncol 2004;26(11):727-734.

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Torben Ek

Department of Pediatrics, Institute for the Health of Women and Children,
The Sahlgrenska Academy at Goteborg University,
The Queen Silvia Children's Hospital, SE-416 85, Goteborg, Sweden

ABSTRACT

Children with acute lymphoblastic leukemia (ALL) can be cured with cytotoxic chemotherapy, but myelosuppression and immunosuppression are major side effects causing morbidity and even mortality from infections. Vaccinations with diphtheria toxoid (DT), tetanus toxoid (TT) and protein conjugated *Haemophilus influenzae type B* (Hib) capsular polysaccharide were used to investigate the adaptive immune system in a controlled study of 31 children after treatment for ALL. Subprotective antibody levels were found in 83% of the patients against diphtheria and 67% against tetanus, whereas all had protective levels of Hib antibodies. All standard and intermediate risk patients had protective antibody levels after immunization. The memory response was weak in the high risk (HR) group, with subprotective antibody levels in a substantial proportion after immunization. Antibody avidity after immunization was low for anti-TT, but not for anti-Hib, in the HR group. The poor antibody production in the HR group correlated to low numbers of specific antibody secreting cells after immunization. No difference in the immune response was detected between patients vaccinated at one month (N=12) or six months (N=19) after treatment. To examine immune reconstitution after childhood ALL, lymphocyte populations and *in vitro* function of T and B cells was measured in the vaccine recipients. At 6 months after treatment T cells were subnormal due to low $CD4^+$ and $CD4^+45RA^+$ T cells. During reconstitution the $CD5^+$ B cells were increased, most marked in the HR group. These findings clearly suggest a relationship between treatment intensity and immunosuppression in children with ALL, which should influence the policy for immunizations. Inactivated vaccines are effective in patients from the lower risk group already at 1 month after treatment. The effect of repeated immunizations after 6 months in the HR group should be examined.

Ara-C is an important, but highly myelosuppressive drug for ALL. To investigate the inflammatory reaction named the Ara-C syndrome a retrospective study of 57 patients in first complete remission (ALL=49, NHL=8) treated with 169 courses of high dose ara-C (HDAC) was performed. Ara-C fever occurred in 113/169 (67%) of the courses, and was associated with elevated plasma levels of the inflammation markers CRP and procalcitonin. An association between fever and release of proinflammatory cytokines (TNF-α, IL-6 and IFN-γ) was found. This was counterbalanced by elevations of the anti-inflammatory cytokines IL1-ra and IL-10. The syndrome was self limiting, but could be inhibited by administration of corticosteroids. Myelosuppression, including lymphopenia, was profound after HDAC, and neutropenic fever occurred after 55% of the courses. The incidence of viridans streptococcal sepsis was low (2/169) and no mortality occurred, despite that 93% of all HDAC was administered without the use of colony stimulating factors. This study demonstrates that ara-C has strong effects on the innate immune system leading to an exceptionally high incidence of both drug fever and infections.

Key words: acute lymphoblastic leukemia, immune reconstitution, immunization, antibody, avidity, ara-C, fever, cytokines

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Knowing what
thou knowest not
is in a sense
omniscience.

Piet Hein

CONTENTS

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ABBREVIATIONS

AbSC	Antibody secreting cells
a-CD3	Antibody to CD3
ALC	Absolute lymphocyte count
ALL	Acute lymphoblastic leukemia
ANC	Absolute neutrophil count
ConA	ConcanavalinA
CRP	C-reactive protein
DT	Diphtheria toxoid
G-CSF	Granulocyte Colony Stimulating Factor
HDAC	High dose Ara-C
Hib	Hemophilus influenzae type B
HR	High risk
IFN	Interferon
IL	Interleukin
Ig	Immunoglobulin
IR	Intermediate risk
NHL	Non-Hodgkin lymphoma
NOPHO	Nordic Society of Paediatric Hematology and Oncology
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
PCT	Procalcitonin
PHA	Phytohemagglutinin
Plt	Platelets
SR	Standard risk
TNF-α	Tumor necrosis factor-α
TT	Tetanus toxoid
VS	Viridans streptococci (α-hemolytic streptococci)
WBC	White blood cells

INTRODUCTION

Childhood acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL), originating from a clonal proliferation of lymphoblasts in the bone marrow, is the commonest pediatric malignancy. The annual incidence rate in children 0-14 years old in the Nordic countries is ≈ 4 / 100 000 (1). The 5 year survival rate has increased from ≈ 5% in 1970 to 81% in the Nordic ALL protocol of 1992 (NOPHO-92) (2).

Much progress has been accomplished through the insight that ALL is a heterogenous disease. This has led to adaptation of therapy depending on the presence of the most important prognostic factors and assignment of patients to different risk groups. An attempt to agree on universal risk criteriae was done in the NCI/Rome classification of 1996 (3). Although all modern treatment protocols for childhood ALL stratify therapy according to such risk groups, the precise definitions of these vary beween different studies. The most important risk factors at diagnosis are white blood cells (WBC) count, age and immunophenotype. Besides, specific cytogenetic abnormalities, treatment and response to treatment are well defined risk factors nowadays. In NOPHO-92, classification into 3 main groups was based on these factors (table 1) (4). The proportions of patients were standard risk (SR) 35%, intermediate risk (IR) 37% and high risk (HR) 29%. The HR group was subdivided in 3 groups: (1) HR <5 y (2) HR

Table 1. *Risk classification in the NOPHO-92 protocol*

Risk group	Age (y)	WBC ($\times 10^3/\mu L$)	Immuno Phenotype	Other criteria
Infants (excluded)	<1	-	-	-
B-ALL (excluded)	-	-	Mature B	-
Standard risk	2-10	<10	B-precursor	No HR-crit
Intermediate risk	1-2	<50	B-precursor	No HR-crit
	2-10	10-50	B-precursor	No HR-crit
	>10	<50	B-precursor	No HR-crit
High risk	>1	-	T-precursor	-
		>50	B-precursor	-
		-	-	CNS+, testis+
		-	-	Lymphomatous (clinical and laboratory crit)
		-	-	t(9;22) / t(4;11)
		-	-	Slow response to induction treatment

(- denotes any value)

≥5 y (3) Very HR, that included children ≥5 y and CNS+ or lymphomatous leukemia or slow response to treatment or T precursor disease together with another HR criteria (5).

Intensive chemotherapy is required to cure childhood ALL. In NOPHO-92 a common induction phase was followed by an early intensification in the IR and HR groups (table 2) (4, 5). Thereafter CNS-consolidation, delayed intensification (the IR and HR groups) and maintenance therapy subsequently followed. To reduce the number of children exposed to cranial irradiation, CNS-consolidation was based on intrathecal methotrexate and high doses of methotrexate iv (5 – 8 g/m^2) and Ara-C iv (2 × 2 g/m^2 for 3 days = 12 g/ m^2). Only very HR patients were irradiated. The NOPHO strategy was successful and the cumulative incidence of CNS-relapses was < 5%, despite that only 10% of all patients (33% of the HR group) received cranial irradiation (6). A few patients with very HR ALL had allogeneic stem cell transplantation in first remission, but this was not an integrated part of the protocol.

Given the myelosuppressive effects of ALL and its treatment, infectious complications are common and supportive care is an essential part. The description of the strong association between neutropenia and infections was a landmark in management of infections (7). However, the association between leukemia and immunological dysfunction has also been known for a long time (8-10). Already in 1920 Dr Katharine M. Howell described failure of humoral immunity in leukemia patients. She examined two patients with untreated leukemia, who failed to form any agglutinating or opsonizing antibodies after inoculation with B. typhosus or B. paratyphosus (8).

Immunosuppression and immune reconstitution

Immunosuppression, which can be caused both by leukemia and its treatment, is of major importance for pediatric cancer patients. Suppression of the innate immune response, e.g. neutropenia and monocytopenia, causes an increased sensitivity for bacterial and fungal pathogens (11). The adaptive immune system is also affected by therapy, but due to more complex interactions, the consequences are more difficult to predict. The risk of opportunistic infections with viruses, particularly of the herpesvirus and paramyxovirus groups, and intracellular organisms like pneumocystis carinii, as well as infections with encapsulated bacteria is increased (12-17). This

broad range of infections implies deficiencies of both T and B cell function (18-20). Thus, a correlation between low levels of $CD4^+$ T cells and opportunistic infections has been found for pediatric patients with solid tumors (21). A single center study found that children with HR-ALL treated with the NOPHO-92 protocol had a cumulated risk of 70% for pneumocystis pneumonia, and that the risk correlated to lymphopenia (22).

Immune reconstitution is an active process by which an organism strives to restore function when the homeostasis of the immune system has been disturbed. To some extent regeneration occurs already between cytotoxic courses, but in general immune reconstitution refers to recovery after end of treatment (23). Some basic principles of immune reconstitution are known (figure 1). The innate immune system regenerates more rapidly than the adaptive immune system, and B cells faster than T cells. Expansion of peripheral T cells precedes the reconstitution of naïve T cells, which require thymic maturation.

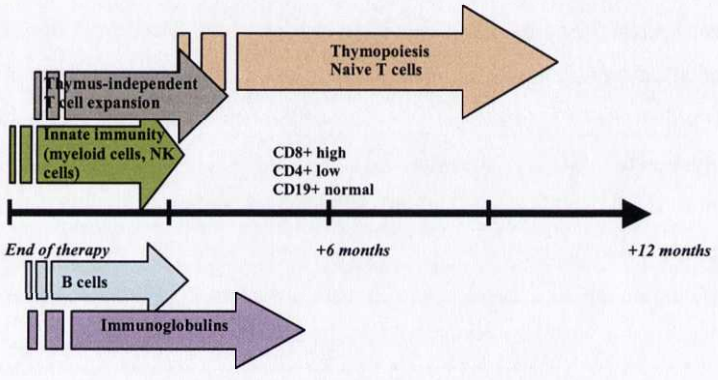

Figure 1. *Schematic time scale of the immunological reconstitution after intensive chemotherapy in children.*

Regeneration of T cells

T cells are depleted during intensive chemotherapy (21, 23, 24). Mackall et al have studied the regeneration of lymphocyte subpopulations in pediatric patients, treated with intensive chemotherapy for solid tumors. They found that the $CD4^+$ T cell regeneration in children is related to thymic function, consequently declining after puberty (25). The median time to full regeneration was 8 months (range 6-12) after

Table 2. *Overview of the NOPHO-92 protocol*

SR (2.5 y)	**Induction** Pred, Vcr, Doxo, L-Asp Mtx IT		**Consolidation** HDM × 3		**Maintenance** Oral Mtx / 6-MP HDM × 5 Vcr/Pred × 6					
IR (2 y)	**Ind 1** Pred, Vcr, Doxo, L-Asp, Mtx IT	**Ind 2** Cyclo, Ara-C, Oral 6-MP, Mtx IT	**Consolidation** HDM × 4 Oral 6-MP		**Reind 1** Dexa, Vcr, L-Asp, Dauno	**Reind 2** Cyclo, Ara-C, Oral TG, Mtx IT	**Maintenance** Oral Mtx / 6-MP HDM × 5 Vcr/Pred × 4			
HR (2 y)	**Ind 1** Pred, Vcr, Doxo, L-Asp, Mtx IT	**Ind 2** Cyclo, Ara-C, Oral 6-MP, Mtx IT	**Cons 1** HDM × 2 HDAC × 2	**Interim maintenance** Oral Mtx / 6-MP Vcr/Pred × 2	**Reind 1** Dexa, Vcr, L-Asp, Dauno	**Reind 2** Cyclo, Ara-C, Oral TG	**Cons 2** HDM × 1 HDAC × 1	**Interim maintenance** Oral Mtx / 6-MP Vcr/Pred × 2	**Cons 3** HDM × 1 HDAC × 1	**Maintenance** Oral Mtx / 6-MP Vcr/Pred × 5 Mtx IT
							Very HR: CNS irradiation 18 Gy and LSA2L2			

Pred=Prednisolone, Vcr=vincristine, Doxo=doxorubicine, Mtx=methotrexate, Asp=asparaginase, Cyclo=Cyclophosphamide, TG=Thioguanine, MP=mercaptopurine, Dexa=dexamethasone, Dauno=daunorubicine, ith=intrathecally

completion of treatment. In contrast, $CD8^+$ T cells, which regenerated primarily through thymus-independent pathways reached pretreatment values at +3 months (26).

T cell recovery after ALL was studied by Alanko et al (27). They found that the total T cell number recovered in 6 months, $CD8^+$ T cells reached normal levels at +3 months, and $CD4^+$ T cells at +6 months. As for children with solid tumors, young children (3-6 years old) had a quicker recovery. T cell defects may be long standing, as shown in a study where 4% of long-term survivors had $CD4^+$ lymphocytopenia after > 5 years in remission from ALL or non Hodgkin lymphoma (NHL) (28).

T cell function is also affected by ALL treatment (29). The proliferative capacity after PHA-stimulation was reduced during induction therapy, but restored in the maintenance phase. More refined measures, like stimulated IFNγ-secretion and cytotoxic respone to allogeneic cell stimulation were reduced during all phases.

Regeneration of NK cells

NK cells are reduced during ALL treatment, whereas they seem to be normal during therapy for solid tumors (21, 30, 31). Following ALL treatment, NK cells regenerate in the first months (31). Recovery of NK cells is important not only for host defense against infections but may also influence the risk of leukemic relapse, since it has been shown that reduced NK cell reactivity against autologous leukemic blasts correlates to increased risk of relapse (32).

Regeneration of B cells

Humoral immunity is affected during ALL treatment with profound B cell lymphopenia and low immunoglobulin (Ig) levels, particularly IgM (33-35). Few data exist, but one study found that B cells increased rapidly to normal levels already at +1 month, whereas serum Ig levels increased at a slower pace. At +6 months IgG, IgA and IgM were normal, but some young patients (<8 y) had persistently low IgG2 (34). After intensive treatment for solid tumors the B cells increased to 150-200% of normal values at +3 months (26). Subpopulations of B cells have not been extensively studied, but it has been shown that $CD27^+$ B cells (memory B cells) are decreased during recovery from ALL treatment (36). Humoral immunity may be affected for long periods after ALL, since some children fail to respond to vaccination with

common vaccines against bacterial and viral infections several years after treatment (37, 38).

Vaccine induced immunity after ALL

Immunity and protection

Protective immunity is a relative concept, and depends on many factors related to the host and the pathogen. The first defense line of adapted immunity against invasive infection or toxin-mediated disease is opsonizing or neutralizing antibodies produced by plasma cells in the bone marrow (39). The memory response, or recall antibody response, is activated after a short lag period. It is characterized by a rapid production of high-affinity antibodies and depends on both memory B and memory T cells. The generation of non-secreting memory B cells takes place in the germinal centres (40). Both measureable antibody levels and memory B cells can persist for very long times after antigen exposure (41, 42).

Vaccine efficacy is demonstrated by a correlation between vaccination and disease prevention (43). To determine protection at the individual level measures of an immune response are used. Neutralizing antibody tests evaluate the level of functional antibodies, and are considered to be the best serological correlates to protection (44-47). ELISA methods are often used for antibody analysis, despite an inherent sensitivity to antibody affinity (48). They tend to overestimate the amount of protective antibodies, since low-affinity antibodies, that confer less protection especially at low levels, also bind in the assay (44, 46). There is some inconsistency regarding protective antibody limits, but when ELISA methods and international standards are used, anti-diphtheria toxoid (DT) / anti-tetanus toxoid (TT) >0.1 IU/ml is considered to represent complete protection against diphtheria or tetanus (44, 49-51). Neutralizing/opsonizing IgG antibodies to Haemophilus influenzae type B (Hib) capsule polysaccharide are essential for the defense against invasive infections. The question about serological correlates to protection from Hib has attracted much dispute. The currently used limit for protection is >0.15 μg/ml (52). In studies of pure polysaccharide vaccines a postimmunization level >1.0 μg/ml was the limit for good immune response, probably conveying long term immunity (49). Protein-conjugated vaccines augment protection by inducing T cell dependent immunological memory.

Antibody avidity

Functional antibody activity is dependent upon both amount of antibody and affinity to the antigen. Analysis of avidity has been developed as a method of measuring the average binding strength of antibodies in a serum sample (53, 54). In vivo, it appears that antibody amount is correlated to protection, provided that avidity is above a certain threshold (55). It has been suggested that avidity can be used as a surrogate marker of memory responses (56). Goldblatt et al found that children with suboptimal levels of anti-Hib IgG also had low avidity, indicating insufficient memory priming (57). Few and conflicting data exist about antibody avidity after childhood ALL. Abrahamsson et al found that the avidity of E. Coli and poliovirus antibodies was increased in ALL patients (58). Nilsson et al studied the immune response to revaccination with live measles vaccine >2 years after treatment and found that children with low antibody levels after vaccination also had low antibody avidity (37).

Immunity after ALL treatment

Why is it of importance to evaluate the immunological protection against pathogens after cancer therapy? Although it must be held in mind that there is no herd immunity for tetanus and occasional case reports exist, this disease is not seen as a clinical problem for ALL patients in countries with good vaccination coverage (59). Large outbreaks of diphtheria have occurred in eastern Europe, and occasionally also in Sweden (60, 61). Immunosuppressed individuals are at increased risk. Children with leukemia have been reported as a risk group for invasive Hib infections, even at age >5 years (16, 17). Despite the introduction of general Hib vaccination (1992 in Sweden) immuncompromised children may still be at risk, due to vaccine failure (62, 63). Viral agents, like measles and varicella, are causing significant morbidity and mortality in children with leukemia (12, 14, 15, 64).

Measurement of vaccination antibodies is an accessible way of evaluating the humoral immune function in immunodeficiency states (65, 66). As discussed above, useful methods for evaluation of the important immunological memory function are analysis of: (1) Antibody levels and isotypes after immunization (2) Antibody avidity (3) Specific antibody secreting cells (AbSC) as measure of memory B cells.

A number of studies have examined immunity after childhood ALL (16, 37, 67-75). They cannot easily be compared, due to differences in use of methods for antibody analysis, definitions of protection, treatment protocols, pretreatment vaccination

schemes, vaccines and evaluation at different time points during or after treatment. Some risk factors for losing antibody protection can be discerned: (1) Younger age correlates with risk of losing protection (37, 71, 74). (2) Patients with hematological malignancies seem to be at greater risk than patients with solid cancers (72, 76). However, there are studies that have failed to show a difference between patients with ALL and solid tumors (71, 74). (3) Recent studies show a higher proportion of patients without protection than earlier studies, probably reflecting higher treatment intensity (figure 2).

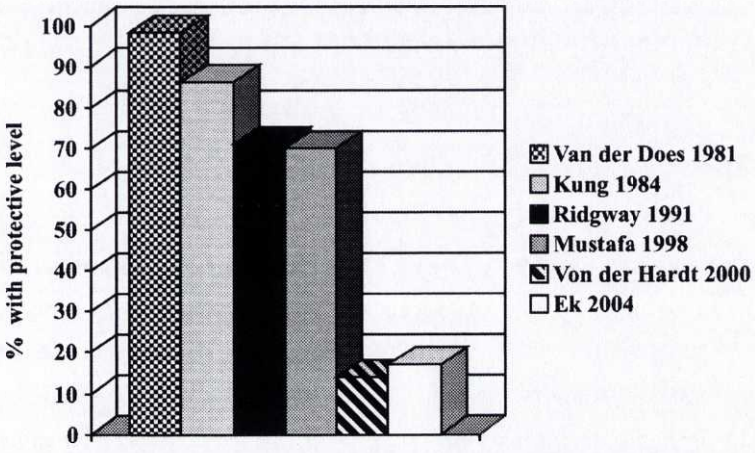

Figure 2. *Proportions of children with ALL with protective levels of diphtheria toxoid antibodies in different studies published over the years 1981 – 2004. Adapted from the references (67-69, 71, 72, 77)*

In general, antibodies against DT and TT decrease during treatment and do not increase spontaneously during the first year after treatment. Nevertheless, the majority (60-100%) of the patients still have antibody levels above the limit for protection after treatment (67-69, 73-75, 78). Exceptions exist, like von der Hardt's study showing that only 14% of ALL patients had full protection against DT after treatment (72). The response to reimmunization has been good in 90-100 % of the patients (67, 69, 73, 74, 78).

A number of investigations have been performed on children with ALL before the introduction of Hib-vaccination (16, 69, 79, 80). They all showed that >50 % of the

patients had subprotective levels of Hib antibodies and that the immune response to vaccination during treatment was variable, with 50-100 % responders.

High-dose Ara-C treatment

Ara-C is a structural analogue of deoxycytidine that is used in the treatment of leukemia and lymphoma. The active metabolite Ara-CTP is incorporated into DNA-strands during replication, leading to DNA-strand breaks and eventual induction of apoptosis (81). Drug concentration and exposure time are important determinants of cytotoxicity. HDAC is used to overcome cellular drug resistance and to achieve therapeutical drug levels in CNS (82). HDAC is extensively used for treatment of AML, and has also been used both for consolidation in primary treatment and relapse of ALL (83, 84).

Toxicity

The commonest toxic side effects in children after HDAC are myelosuppression and mucosal injury in the gastrointestinal tract (85). Neurotoxicity, especially cerebellar dysfunction, is a serious event that is uncommon in pediatric patients (85, 86). A severe respiratory failure with features of acute respiratory distress syndrome (ARDS) and high mortality was first described in adults, but has subsequently also been reported in pediatric patients with AML (87-89). The reaction occurs after an interval of approximately 1 week after HDAC and in most patients coincides with myelosuppression and gastrointestinal damage. The pathogenesis is not fully known, but capillary leakage due to fluid overload, hypoalbuminemia and endothelial injury has been proposed. One study suggested that elevated levels of proinflammatory cytokines, TNF-α and Platelet activating factor, may be important mediators (90).

Since HDAC induces severe myelosuppression, neutropenic infection is a common life threatening complication in pediatric patients (84, 85). In a study of ALL patients, it was found that HDAC + L-asparaginase was followed by the highest rate of neutropenic infections, even when comparing with other treatment blocks inducing similar duration of leukopenia (91). Several studies has shown HDAC to be a risk factor for viridans streptococcal (VS) sepsis, which is a serious complication sometimes evolving to shock and ARDS (92-94). The oral cavity is the commonest

portal of entry, but it has been suggested that Ara-C is an independent risk factor apart from its association with mucositis (94).

The Ara-C syndrome

A remarkable side effect of Ara-C is that a substantial proportion of patients develop systemic inflammatory symptoms during treatment. The reaction resembles the systemic inflammatory response syndrome (SIRS) and may easily be confused with fever caused by infections (95). The term "Ara-C syndrome" was coined by Castleberry et al (96). In their work 6 children exhibited fever, myalgia, bone pain, occasionally chest pain, maculopapular rash and conjunctivitis 6-12 hours after low-dose Ara-C injections. The reaction was self-limiting, but could be inhibited by corticosteroids. They concluded that the most likely explanation was hypersensitivity to Ara-C.

AIMS OF THIS STUDY

The aims of the study were to investigate:

the immunity to diphtheria, tetanus and Hib after treatment for childhood ALL.

the immune response after immunization with DT, TT and Hib after treatment for childhood ALL, and to compare reimmunization at 1 or 6 months after treatment.

the reconstitution of the adaptive immune system after treatment for childhood ALL, with particular focus on finding variables that are predictive of the immune response to reimmunizations.

the incidence and characteristics of the acute inflammatory reaction that is associated with Ara-C treatment, and if proinflammatory cytokines act as mediators.

the myelosuppression and the subsequent infectious complications after single-drug, high dose Ara-C for pediatric lymphoid malignancies

PATIENTS AND METHODS

Patients

Göteborg is the referral centre for pediatric oncology in the western region of Sweden. The mean population for the time period 1993-2002 was 530 000 children (0-18 years). All subjects in the studies are from the 231 new cases of ALL and 13 cases of non-B NHL stage III-IV during the period 1993-2004. The characteristics of cases and controls are shown in table 3.

Table 3. *Characteristics of study I-V*

Study	Time period	Diagnosis	N	Age* (years) Mean(range)	Study design
I-III	1997-2002	SR ALL	6	9.9 (3.3-19.1)	Consecutive patients at 1 or 6 months after treatment
		IR ALL	16		
		HR ALL	9		
		Controls (I)	20	11.1 (5.6-15.7)	Age-matched, healthy controls
		Controls (II)	18	11.4 (7.1-15.7)	
		Controls(III)	40	7.8 (2.1-15.7)	
IV	1993-2004	HR ALL	49	7.2 (1.8-17.8)	Retrospective study
		NHL	8	11.6 (5.6-17.8)	
V	1995- 1998	HR ALL	14	6.3 (2.5-14.8)	Consecutive patients during HDAC treatment
		NHL	2		

*Age refers to age at investigation

In study I-III we included 31 children with ALL in first complete remission and 20 (18 in study II) healthy, age-matched controls. Patients that completed ALL treatment during the study period, and agreed to participate, were alternately included at 1 (N=12) or 6 months (N=19) after treatment. The groups are unequal, because more patients refused to participate in the 1 month group. The slow enrolment was caused by a high proportion of patient and parent refusal and to initial restriction to patients living near Göteborg due to transportation problems. Later we used taxi transports from hospitals outside Göteborg to ensure delivery within 6-8 hours.

The detailed characteristics of the HR patients are shown in table 4. The controls were recruited among healthy siblings and friends of the patients. Patients and controls were vaccinated once with 0.25 ml Duplex® [vaccine against diphtheria (30 Lf/ml) and tetanus toxoids (7.5 Lf/ml), SBL, Stockholm, Sweden] and 0.5 ml Act-Hib® [Hib capsule polysaccharide (CP) conjugated to tetanus toxoid, Aventis Pasteur, MSD]. Serum was collected before vaccination and 3 weeks later for analysis of level and

avidity of DT, TT and Hib antibodies. All serum samples were stored at -70°C until analyzed. Blood cells were collected at 7 days after vaccination for measurement of total number of AbSC (with unknown specificity) and TT or Hib specific AbSC, since the number is known to be maximal at that time (97). In addition we used serum samples that were frozen at the time of diagnosis, to compare immunity before and after treatment. The subjects that were <10 years old, and had not received the scheduled booster dose of DT+TT at 10 years of age, were advised to refrain from this immunization.

In study III we included the same subjects as in study I-II and an additional 20 controls, that were not vaccinated in the study but were sampled for baseline immunological parameters. Peripheral blood mononuclear cells (PBMC) and serum was sampled before vaccination for analysis of lymphocyte subpopulations, mitogen-stimulated PBMC proliferation, total number of AbSC, serum Ig and subclasses of IgG.

In study IV we included 57 consecutive patients with HR ALL (n=49) or non-B NHL stage III-IV (n=8). They received altogether 169 HDAC courses. Study IV was retrospective. Ara-C fever was defined as body temperature ≥38.0° C for >2 hours between the start of the first and the end of the last Ara-C infusion. Fever occuring after the last Ara-C infusion was included in analysis of post Ara-C febrile episodes.

In study V 14 patients with HR ALL and 2 with T-NHL stage III-IV were consecutively included before a planned HDAC treatment (2 g/m^2 × 2 for 3 days = 12 g/m^2). EDTA-blood was sampled before 1st, 2nd, 4th and 6th infusion. One additional sample was drawn if fever occurred. All the samples were frozen for later analysis of cytokines and PCT.

Table 4. *Characteristics of the HR patients in study I-III*

Patient n:o	Sex	Age (years)	Blast phenotype	Special risk Factors	Therapy	Timepoint of study (post-treatment)
1	F	3.8	B-lineage	CNS+	CT	1 month
2	M	4.6	B-lineage	Slow response	CT	6 months
3	M	5.0	B-lineage		CT	6 months
4	F	5.1	B-lineage		CT	6 month
5	M	5.4	B-lineage		CT	1 month
6	M	5.8	T-lineage	Mediastinal+	CT	6 months
7	F	6.9	T-lineage	Mediastinal+	CT+CNS irrad	1 month
8	M	11.7	T-lineage		CT	6 months
9	F	16.5	B-lineage	Lymphomatous	CT	6 months

CT = chemotherapy

Study I-III and V were approved by the local ethics committee of Goteborg University. All patients and controls received oral and written information before consent. Study IV was performed within the framework of the NOPHO-ALL 92 study.

Laboratory methods

Cytokine analyses

Cytokine analyses were performed with commercially available enzyme amplified sensitivity immunoassays (EASIA™, Medgenix, BioSource, Europe) utilizing double monoclonal antibodies. The detection limits were tested by the manufacturer: TNF-α 3 pg/ml, IL-6 2 pg/ml, IFN-γ 0.03 IU/ml, IL-1β 2 pg/ml, IL-8 0.7 pg/ml, IL-10 1 pg/ml, and IL1-ra 4 pg/ml.

Procalcitonin analysis

PCT was analyzed with an immunoluminometric assay (LUMItest PCT®, B.R.A.H.M.S, Hennigsdorf, DE). The sensitivity was given by the manufacturer as 0.1 ng/ml. The reference values for normal individuals is <0.1 ng/ml , and 0.1 - 0.5 ng/ml can be seen in mild infections and inflammatory conditions (98, 99). PCT >0.5 ng/ml is often regarded as suggestive of a systemic inflammatory response, also in neutropenic patients (99-101).

ELISA for detection of specific antibodies

Tetanus toxoid (TT, Statens Serum Institut, Copenhagen, Denmark) in concentration of 5 μg/ml or Diphtheria toxoid [DT, 02/176, National Institute for Biological Standards and Controls (NIBSC), UK] in concentration of 0.5 Lf units/ml were used as antigens and diluted in PBS for coating of microtiter plates. After washing with PBS the plates were blocked with 5% fish gelatine in PBS for 1 hour at room temperature. All additional washings were with PBS-0.05% Tween 20 (PBS-T). International Standard for Tetanus Immunoglobulin, Human (TE-3; 120 IU/ml) or Diphtheria antitoxin, human serum (00/496; NIBSC; 1.6 IU/ml) were used as reference in the IgG antibody assay. Alkaline phosphatase-conjugated rabbit-anti human IgG 1:3000 (DAKO) and 1 mg/ml of AP-substrate o-nitrophenyl-b-D-

galactopyranoside (Sigma) in 1M diethanolamine buffer pH 9.8 (DEA) were used for detection. The reference for TT was used in 8 three-fold dilutions from 0.4 IU/ml → 0.00017 IU/ml and for DT in eight 2,5-fold dilutions from 0.08 → 0.0001 IU/ml for construction of standard curves. All reference preparations, serum samples and conjugates were diluted in PBS-T. Serum samples were analyzed in dilutions of 1:100 and 1:100 000 (tetanus) or 1:100 and 1:10 000 (diphtheria) to be certain that the whole range of values was covered by the standard curve, and to assure that the antibodies could be diluted out after a high dilution to undetectable levels.

TT specific IgA and IgM antibodies were analyzed with use of the same antigens as in the IgG assay. As no international standard was available, an internal high titered serum was used as a reference. Serum samples were diluted 1:100 and 1:1000 in PBS-T and the reference serum was diluted in four 10-fold dilutions from 1:100 → 1:100 000. AP-conjugated rabbit anti-human IgA or IgM 1:1000 (DAKO) were used for detection.The patient serum antibody levels were expressed as per cent of absorbance of the reference serum in the same dilution.

In the Hib assays biotinylated Hib-CP diluted in PBS in a concentration of 2 μg/ml was used as antigen after precoating of the plates with 5 μg/ml of avidin (Sigma, USA) diluted in PBS. After washing with PBS the plates were blocked with 5% fish gelatine (Sigma) for 1 h at room temperature. Human anti Hib-CP standard with known concentration of anti-Hib IgG (60.9 μg/ml), anti-Hib IgA (5.6 μg/ml) and anti-Hib IgM (3.5 μg/ml) (Lot 1983 from Laboratory of Standards and Testing DMPQ/CBER/FDA, Bethesda, Maryland) was used as a standard. The standard was diluted in PBS-T-1% fish gelatine in 1:2 steps from 0.2 μg/ml → 0.0016 μg/ml (IgG), 0.056 μg/ml → 0.0004 μg/ml (IgA) and 0.035 μg/ml → 0.0003 μg/ml (IgM). The patients sera, and Anti-Hib Human Reference Serum (96/536, NIBSC) used as positive control, were diluted 1:100 and 1:1000 in PBS-T-1% fish gelatine. The AP-conjugates used for detection were Rabbit anti-human IgG (1:2000), IgM and IgA (1:1000), all from Dakopatts. Antibody levels were expressed in μg/ml.

All assays were performed with duplicates of standards and samples, and the identity of the samples was blinded to the laboratory investigator.

Antibody avidity

The avidity of serum IgG antibodies against Hib-CP and tetanus toxoid was determined using potassium thiocyanate (KSCN) elution, in a modified ELISA described previously (54, 102). The antigen-bound antibodies were eluted with 7 different molarities of KSCN (0.1-10 M). The molarity of KSCN needed to achieve an absorbance value of 50% of the absorbance value without KSCN was determined. The relative avidity index (AI) was expressed as the molarity of KSCN (M) equivalent to the 50% elution point.

Flow cytometry of PBL

Flow cytometry was performed on a FACScan instrument (Becton-Dickinson) as previously described (103). The monoclonal antibodies used were anti-CD3, -CD4, -CD5, -CD8, -CD19, -CD45RA, -CD45RO, -CD56 and -HLA DR (Becton-Dickinson, Mountain View, CA, USA). The following designations were used:

$CD3^+$	T cells
$CD3^+4^+$	T helper lymphocytes
$CD3^+8^+$	Cytotoxic T cells
$CD3^+4/8^+45RA^+$	Naïve subset
$CD3^+4/8^+45RO^+$	Antigen-primed (memory) subset
$CD3^-56^+$	NK cells
$CD3^+56^+$	NKT
$CD19^+$	B cells
$CD5^+19^+$	B-1 subset

ELISPOT methods for enumeration of AbSC

Vaccinations induce both increased circulating AbSC and antibody levels (104, 105). In order to adress the question if low antibody levels are correlated to low number of AbSC the ELISPOT technique was used for enumeration of both total number of AbSC with unknown specificity (IgG/A/M) and TT or Hib specific AbSC (IgG/A/M) (106). PBMC were separated from heparinized whole blood with Lymphoprep (Nycomed Pharma AS, Norway). A single cell suspension of PBMC was washed, resuspended and incubated on a solid phase with or without antigen, followed by an immuno-enzymatic procedure which allowed the visualization of total or antigen-specific B cells as single dark spots. The spots (AbSC/10^6 PBMC) were counted. Total number of AbSC (IgG, IgA or IgM) was determined in unstimulated PBMC,

and after stimulation with Epstein-Barr virus (EBV) or pokeweed mitogen (PWM; EY laboratories Inc. San Mateo, CA, USA) before vaccination. The total number of AbSC and the numbers of TT or Hib specific AbSC of different isotypes (IgG, IgA or IgM) were determined 7 days after vaccination.

Proliferation of PBMC after mitogen stimulation

PBMC were stimulated with PHA (10 μg/mL; Murex Biotech Ltd, UK), ConA (50 μg/mL; Sigma-Aldrich Fine Chemicals, St Louis, MO, USA) or monoclonal anti-CD3 (125 ng/mL; Ortho Diagnostic Systems Inc., Raritan, NJ, USA). Proliferation was measured as incorporation of ^{3}H-thymidine as previously described (103).

Ig and subclasses of IgG

IgG, IgA, IgM and IgG subclasses were analyzed by radial immunodiffusion (103). The concentrations are expressed as g/L.

Statistical methods

In study IV variables were tested for normality and presented as mean and 95% confidence interval if appropriate. In the other studies median and range was used because of small samples with skew distributions. Non-parametric tests were used: The Kruskal-Wallis H test (multiple independent samples), the Mann-Whitney test (two independent samples), or the Wilcoxon signed rank sum test (two related samples) for comparison between groups. The Spearman rank correlation test for correlation between variables. Categorical data were analyzed with chi-square statistics. All p-values are two-sided (107). The statistical analyses were performed with SPSS v 11.0.

RESULTS

Immunity after childhood ALL (study I-II)

Levels of specific IgG antibodies decreased during treatment for childhood ALL (figure 3 and table 5). After treatment, a considerable proportion of the patients lacked complete antibody protection against diphtheria and tetanus, whereas all had protective levels against Hib (table 6).

Table 5. *IgG antibodies (median and range) against DT, TT and Hib in children with ALL. All risk groups are included.*

	At diagnosis (N=31)	After treatment (N=31)	P
Anti-DT (IU/ml)	0.08 (0.01-5.69)	0.06 (0.01-1.24)	0.005
Anti-TT (IU/ml)	0.65 (0.09-5.20)	0.07 (0.01-1.00)	<0.001
Anti-Hib (µg/ml)	5.55 (0.40-36.50)	1.00 (0.20-40.50)	0.001

No HR patients had protection against diphtheria and tetanus, but 9/9 had protection against Hib. The immune response also showed marked differences between the risk groups. All SR and IR patients achieved complete protection against all antigens after vaccination. On the other hand, in the HR group 5/9 were protected against diphtheria, 2/9 against tetanus, and 7/9 against Hib after vaccination (figure 3).

Table 6. *Protective immunity Proportions of patients with full antibody protection against diptheria, tetanus and Hib. The limits for protection are defined as: (1) Anti-DT and anti-TT >0.1 IU/ml (2) Anti-Hib >0.15 µg/ml before and >1.0 µg/ml after vaccination..*

	ALL (N=31)			Controls (N=20)		
Timepoint	**At diagnosis**	**Before vacc**	**After vacc**	**At diagnosis**	**Before vacc**	**After vacc**
Diphtheria	39%	17%	87%	-	45%	100%
Tetanus	81%	33%	77%	-	50%	100%
Hib	100%	100%	93%	-	100%	100%

Neither the immunity, nor the immune response differed between patients vaccinated at 1 month or 6 months after treatment.

IgA and IgM antibodies against TT and Hib were also analyzed, and the results showed that the SR and IR groups had levels that were comparable to the control

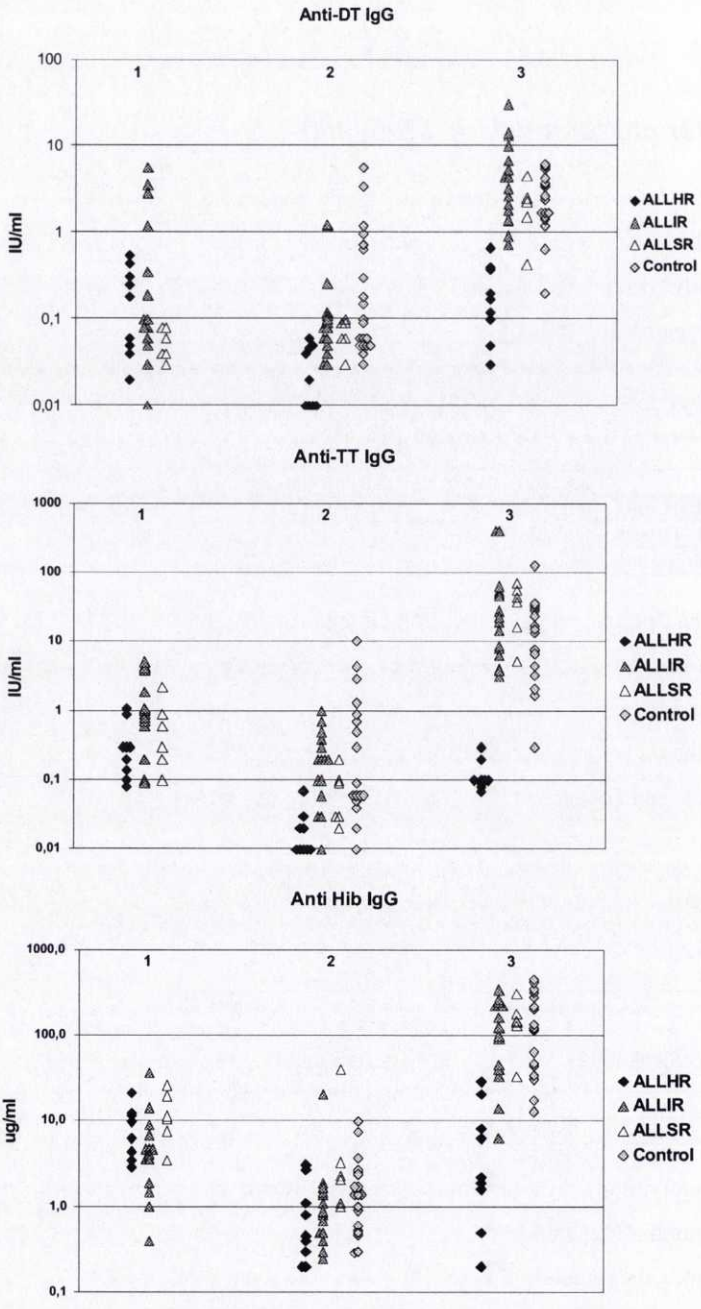

Figure 3. *Specific IgG antibodies against DT, TT and Hib. Antibody level at 3 different time points in children with ALL compared to controls (1 = at diagnosis, 2 = before vaccination and 3 = after vaccination). The values are grouped according to ALL risk group.*

group. The HR group had lower levels of all antibodies after vaccination (IgA_{TT} p=0.002; IgM_{TT} p<0.001; IgA_{Hib} p<0.001; IgM_{Hib} p<0.001).

The total number of IgG AbSC (with unknown specificity) was higher in the HR than the controls before vaccination [440 (200-1600) vs 205 (60-1100) AbSC/10^6 MNC (median and range); p=0.03] and lower in the HR at 7 days after vaccination [300 (20-1400) vs 1000 (300-5200) AbSC/10^6 MNC; p=0.01]. Analysis of TT and Hib specific AbSC in peripheral blood at one week after vaccination showed that the HR group were virtually devoid of TT specific IgG cells and IgA cells (figure 4). The number of TT specific IgM cells was not decreased in the HR group. The Hib specific cells showed a different isotype distribution in SR, IR and controls, with more IgA and IgM cells and less IgG cells than for TT. The HR group showed very few Hib specific AbSC.

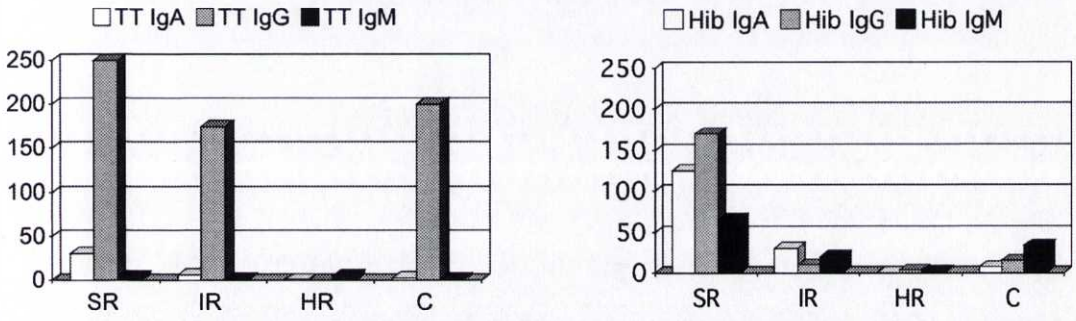

Figure 4. *The median number of TT and Hib specific antibody secreting cells at 7 days after vaccination (AbSC / 10^6 PBMC).*

The median avidity of anti-TT was marginally lower at diagnosis of ALL compared to the control group after immunization. The median avidity of anti-Hib was higher at diagnosis of ALL compared to the control group after immunization (III). Antibody level and avidity correlated for anti-TT IgG after vaccination (r_s=0,59; p<0,001). The HR group displayed both low concentration and low avidity of anti-TT IgG (figure 5). For anti-Hib IgG there was no correlation between concentration and avidity, and no patients were found with low level and low avidity concomitantly.

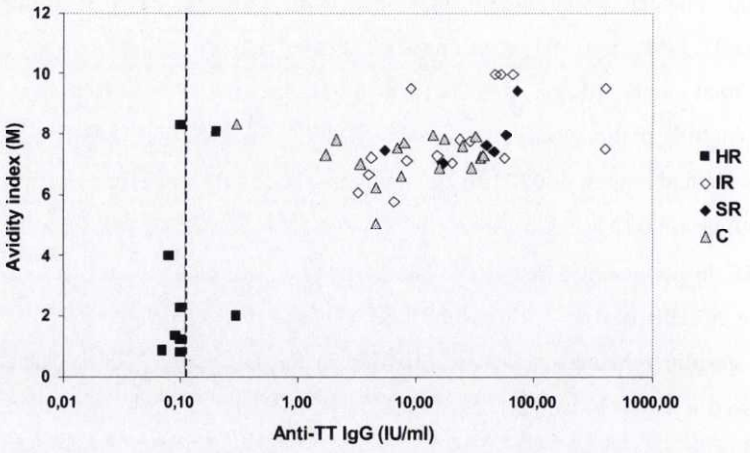

Figure 5. *The relation between antibody level and avidity after vaccination. Levels and avidity of anti-TT IgG at 3 weeks after vaccination in ALL patients, grouped in three risk groups, and controls. The line (- -) indicates the limit for complete protection.*

Immune reconstitution after ALL (study III)

Lymphocyte populations

The different lymphocyte subpopulations were measured at 1 or 6 months after treatment for ALL. The time scale for regeneration varies between the major lymphocyte populations (figure 6). Both the $CD3^+$ and $CD3^+4^+$ were still decreased at 6 months, whereas $CD3^+CD8^+$ were nearly normal both at 1 and 6 months. The $CD4^+/CD8^+$ quotient at 1 month was 1.12 (0.3-1.7; median and range) ($p=0.001$) and at 6 months 1.06 (0.3-2.8) ($p=0.006$) compared to 1.5 (0.8-2.6) in the control group. The $CD4^+45RA^+$ subset was decreased both at 1 and 6 months (figure 6B), and the number of $CD4^+$ at 6 months after treatment was strongly correlated to the number of $CD4^+45RA^+$ ($r=0.93$; $p<0.001$). The $CD19^+$ were normalized at 6 months, but subset analysis showed that this was mainly due to an increase of the $CD5^+19^+$ (B1 B cells), since the $CD5^-19^+$ were subnormal also at 6 months (fig 6C). The median proportion of B1 cells was 64% at 1 month ($p<0.05$), 54% at 6 months ($p<0.001$) versus 38% in the control group. NK cells were low at 1 month, but normal at 6 months (fig 6D). The NKT subset ($CD3^+56^+$) was low both at 1 and 6 months.

Looking closer at the different risk groups revealed some differences between the IR and HR group at 6 months after treatment. Both had decreased T cells, $CD4^+$ T cells (both $CD4^+45RA^+$ and $CD4^+45RO^+$, but the HR group in addition had decreased $CD8^+$ T cells (median 0.32 × 1000/μL; $p<0.05$ vs the control group). The HR group also had the highest number of $CD5^+$ B cells (median 0.33 × 1000/μL; $p<0.05$ vs the control group).

Lymphocyte function and Ig levels

The proliferative capacity of T cells was tested with PHA, ConA and a-CD3 as stimulators. The response to a-CD3 was normal both at 1 and 6 months. The responses to PHA and ConA were both lower at 1 month, but at 6 months only the ConA response was decreased . No differences were detected between the risk groups (data not shown).

B cell function *in vitro* was analyzed as number of AbSC both unstimulated and after stimulation with EBV and PWM. At 1 month after treatment the number of unstimulated IgG and IgA AbSC was increased. At 6 months only IgM AbSC after PWM stimulation was reduced. In the HR group all responses were normal at 6 months.

Total levels of Ig and IgG subclasses showed that IgM levels were lower at 1 month and IgG3 levels were higher both at 1 and 6 months. No significant differences could be detected in total IgG or IgA levels. The HR group had significantly lower IgG2 levels at 6 months posttherapy (0.58 g/L vs 1.6 g/L for controls; $p<0.05$).

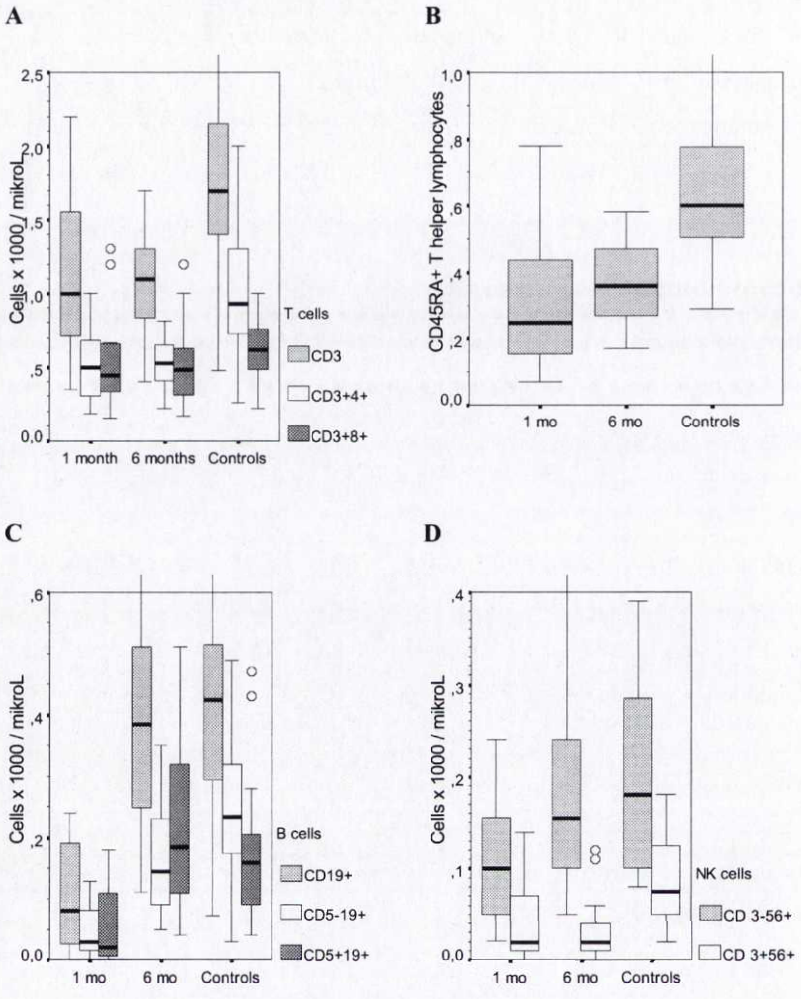

Figure 6. *Reconstitution of lymphocytes after childhood ALL. Lymphocyte subpopulations at 1 month (1 mo; N=12) and 6 months (6 mo; N=19) after treatment for ALL compared to a control group (N=40).* ***A.*** *T cell subpopulations* ***B.*** *$CD4^+45RA^+$ T cells* ***C.*** *B cell populations* ***D.*** *NK cell populations. Median, quartiles (box) and range (whiskers) are shown; ○ = outliers*

Toxicity of HDAC (study IV-V)

The Ara-C syndrome

Fever (>38.0°C) during HDAC occurred in 113/169 (66.9%) courses. The fever began at an average of 26.0 hours ($CI_{95\%}$ 23.7-28.3) after start of the first infusion. The average maximum temperature in the febrile patients during HDAC was 39.1°C ($CI_{95\%}$ 39.0-39.2 and range 38.0-41.0). No septic infections were documented during HDAC, and no patient was febrile at discharge. The inflammatory reaction was accompanied by moderately increased serum levels of CRP. The maximum CRP in the febrile patients was 38.0 mg/L (3-150; median and range).

Corticosteroids were used at the judgement of the physician in charge of the treatment as antiemetic or anti-inflammatory agent. In 16/169 HDAC the patient received steroids already before start of the first infusion ("prophylactic steroids"). In this group only 3/16 (18.8%) developed fever compared to 110/153 (71.9%) of the others ($p<0.001$).

In the subgroup of 16 patients examined for cytokines 13 developed fever. Figure 7 shows the plasma levels of six different cytokines in 16 patients at 4-5 different timepoints during HDAC.

Proinflammatory cytokines: TNF-α increased at 12 h, preceding the fever in all patients. This was followed by increases of IL-6 and IFN-γ, peaking at the onset of fever. Levels declined at the end of the treatment. Levels of IL-8 and IL-1β were low or undetectable.

Anti-inflammatory cytokines: IL-1 receptor antagonist (IL-1ra) increased sharply at the onset of fever. Whereas only 1/16 patients had detectable IL-10 before treatment, 13/16 had detectable, but low, levels of IL-10 at 36 or 60 h ($p=0,05$ and 0,02 respectively).

A correlation was found between the plasma level of IL-6 at 36 hours and the maximum CRP level ($r_s=0.53$; $p=0.04$).

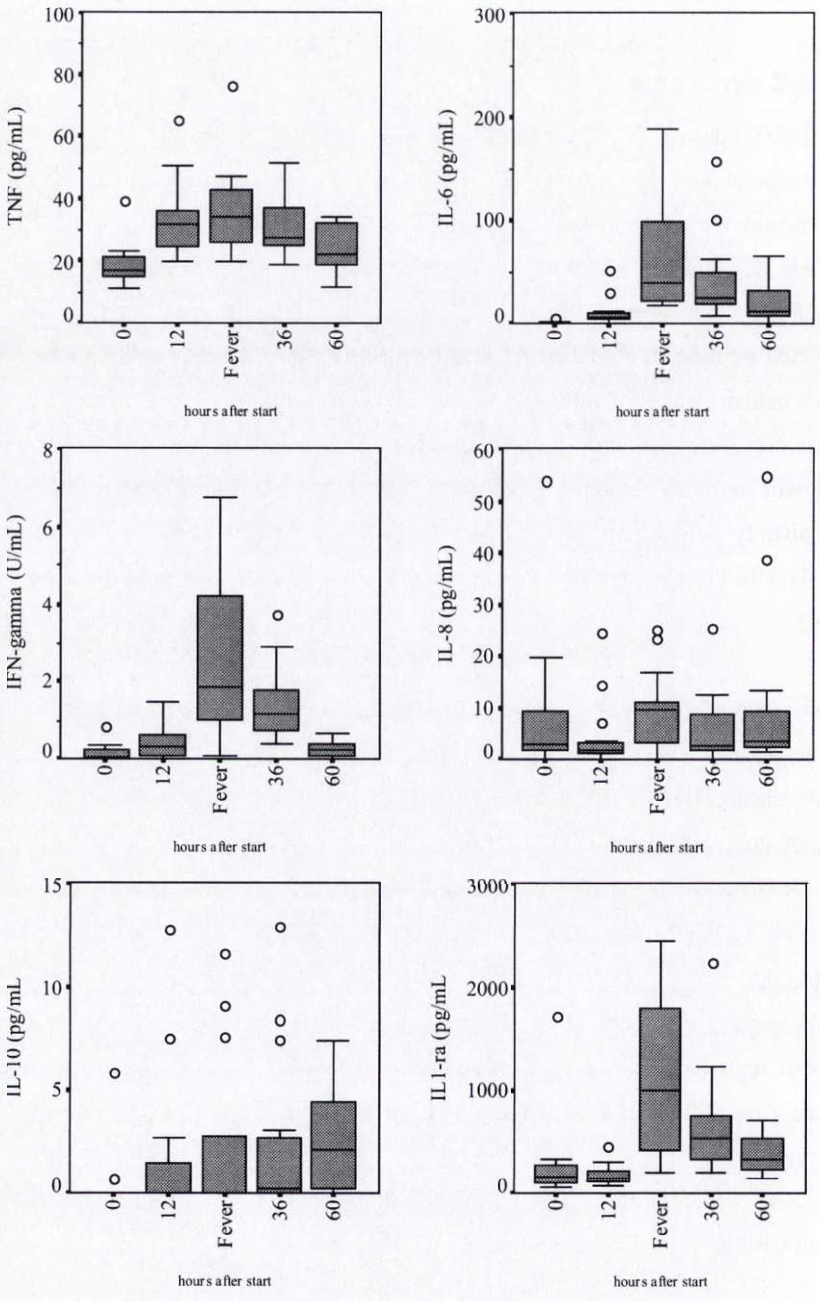

***Figure** 7. Cytokine values during high dose Ara-C treatment. Plasma levels of six cytokines at 4-5 different timepoints during HDAC. 13/16 patients had fever, at a median time point of 28 hours after start (20-47), and for clarity the values at fever is inserted between 12 and 36 hours. Median, quartiles (box) and range (whiskers) are shown; ○ = outliers*

Procalcitonin: PCT was also analyzed at the same timepoints in the 16 patients (figure 8). All except one patient displayed increasing PCT during HDAC, with large interindividual variations. At 36 hours after start 4/16 patients displayed PCT >0,5 ng/mL. PCT_{36} correlated to the maximum CRP (r_s=0.81; p<0.001).

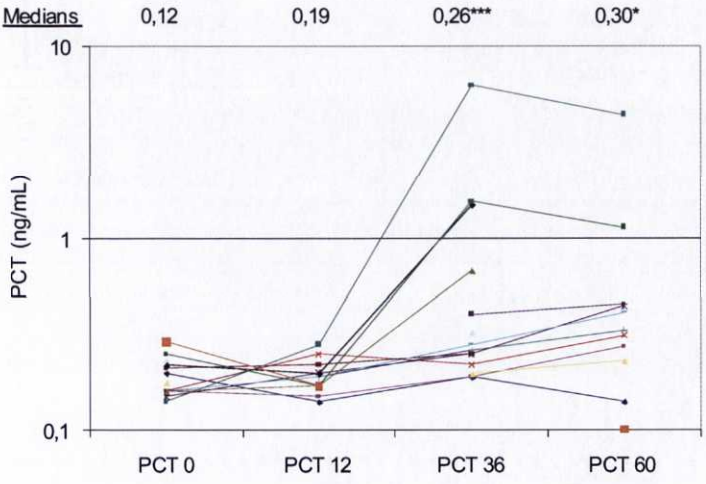

Figure 8. *PCT values at 4 different timepoints during high dose Ara-C. PCT 0 = before start of 1st infusion; PCT 12 = before start of 2nd infusion; PCT 36 = before start of 4th infusion and PCT 60 = before start of 6th infusion. Values at fever are excluded from the figure for clarity Comparisons between PCT 0 and PCT 12/36/60 were made with Wilcoxon signed ranks test. * = p<0.05, *** = p<0.001*

Hematological toxicity

The hematological toxicity was marked and almost universal (figure 9). The nadir of ANC appeared at day +16 (11-24; median and range), and the duration of neutropenia was 11 days (5-18). The nadir of platelets appeared earlier than for neutrophils, at day +13. HDAC induced a rapid reduction of lymphocytes. The ALC decreased at days +1-3 to 0.03 (0.01-0.21) and at days +4-6 ALC was 0.10 (0.01-0.68). In the group that received prophylactic steroids the nadir of WBC was lower [0.4 (0-0.6; median and range) vs. 0.6 (0.1-1.7) in the others] (p=0,001).

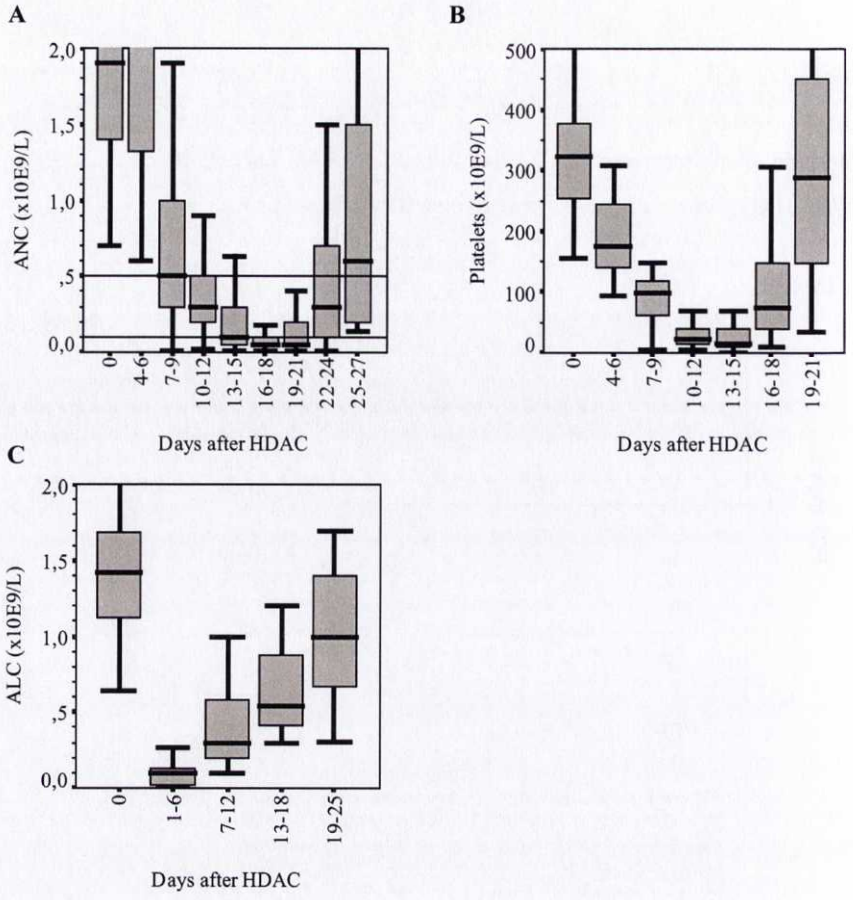

Figure 9. *Blood values after high dose Ara-C.* ***A.*** *Absolute neutrophil granulocytes counts (ANC) and* ***B.*** *Platelet counts (Plt) in 86 cases.* ***C.*** *Absolute lymphocyte counts (ALC) were available in a subgroup of 32 cases. The values in A and B are grouped in intervals of 3 days, and in C in intervals of 6 days. If more than one value/interval was measured, the lowest was included in the analysis. Median, quartiles (box) and range (whiskers) are shown.*

Infections after HDAC

A febrile episode during the period after HDAC occurred after 93/169 (55%) HDAC. The onset of fever was at day +16 (7-23; median and range) and mirrored the nadir of ANC. Only 6/93 infections started before day +10. The cause of fever was septicemia or focal infections in 53/93 (57%), and unknown in 40/93 (43%). The blood culture isolates were gram-positive bacteria in 14 cases and gram-negative bacteria in 2 cases. In 21 cases the infection was related to the central venous catheter, with clinical signs of tunnel infection. No patient needed intensive care and all patients recovered without complications. Two patients had viridans streptococcal sepsis, but they were not

seriously ill. Prophylactic G-CSF was administered after 12/169 (7.1%) in selected cases. A trend towards more infections occurred in the G-CSF group (9/12) than in the non-G-CSF group (82/155) ($p=0.14$). When comparing the frequency of infections after the first HDAC (to avoid multiple inclusions of single patients) it showed that infections were a little more common after Ara-C fever [26/36 (72%)] than after non-febrile Ara-C courses [10/21 (48%)] ($p=0.06$).

DISCUSSION

Immunosuppression and immune reconstitution

There are large individual differences in the frequency of toxic side effects after chemotherapy. Several drugs show a relationship between cumulative dose and side effects: Anthracyclins – cardiotoxicity, alkylators – gonadal toxicity, ifosfamide – nephrotoxicity (108-110). However, a clear correlation between chemotherapy doses and duration and degree of immunosuppression has not been established. The studies I-III strongly suggest a relationship between treatment intensity and immunosuppression in children with ALL. The risk groups showed no major differences in antibody levels or avidities before the initiation of treatment, but afterwards the HR group had more pronounced immune abnormalities than the SR or IR groups, especially for measures of the antigen specific immune response (table 7). The HR group displayed low antibody levels after immunizations, low avidity of anti-TT and low number of TT- and Hib specific AbSC as evidence of a defective immunological memory response.

Table 7. *Schematic overview of the immunological defects in the different risk groups.*

		SR + IR	HR
Antigen specific functions	Antibody levels at diagnosis (DT, TT and Hib)	N	N
	Antibody avidity at diagnosis (TT/Hib)	N/H	N/H
	Antibody levels after treatment	L	L
	Antibody levels after immunization	N	L
	Antibody avidity after immunization (TT/Hib)	N/N	L/N
	TT and Hib specific AbSC	N (H in SR)	L
	Total Ig and IgG subclasses	N (H in SR)	N
	B cells	L	H ($CD5^+$ B)
	Unspecific B cell function	N (H in SR)	N
	T cells	L	L
	Unspecific T cell function	N	N

L = low, N = normal, H = high

Also, reflecting that treatment may affect the immune system in a diverse and complex pattern, the SR group shows signs of immunological hyperactivity. In this group, the antigen specific immune responses were normal, both measured as antibody levels and avidities. Furthermore, the B cell function measured as number of AbSC (both unspecific and TT-/Hib-specific) and serum levels of IgG and IgA were increased. The

reason for this increased responsiveness is unclear, but a reduction and inhibition of regulatory T cells may be induced by less intensive cytotoxic chemotherapy (111).

Table 7 is simplified but nevertheless shows that the major lymphocyte subsets and antigen independent T and B cell function *in vitro* only differ slightly between the treatment groups despite the qualitative differences in the response to immunizations. What mechanisms can explain this apparent discrepancy?

Defects in humoral immunity

A model for the development of low levels of specific antibodies after cytotoxic chemotherapy is proposed in figure 10. Intensive chemotherapy for ALL is toxic to B cells, and induces a reduction of B cells in peripheral blood and plasma cells in the bone marrow (33, 34, 37, 112). This results in decreased serum levels of Ig (19, 35). We propose that specific antibodies decline at a faster rate, and often reach low ("subprotective") levels.

A booster immunization normally activates the memory response resulting in rapid production of high affinity, isotype-switched antibodies (113). We find that the antibody response in SR+IR is quite comparable to the control group, measured both as levels and avidities (I-II). The HR group displays weak antibody responses after booster immunizations, resulting in subprotective antibody levels.

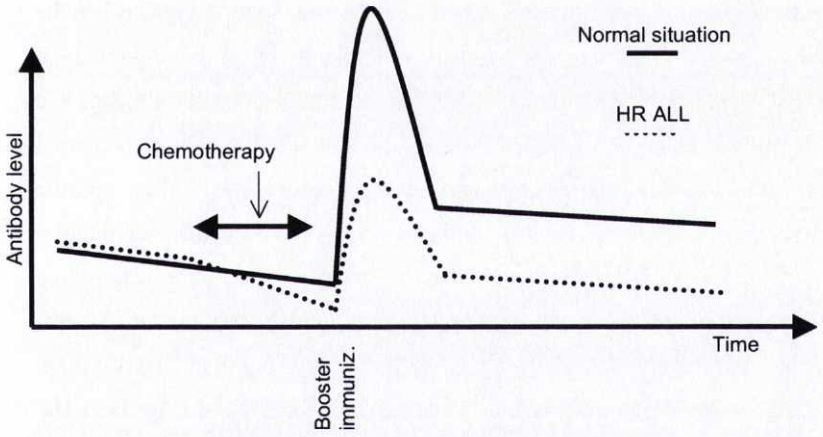

Figure 10. *A proposed model of how ALL patients become deficient of specific antibodies. The black line represents the normal situation, in which serum levels of specific antibodies (e g anti-TT or DT) are maintained both through continous antibody production by long lived plasma cells in the bone marrow and polyclonal activation of memory B cells (114, 115). A booster immunization activates memory B cells and leads to an antigen-driven increase of specific antibodies. The dotted line represents what happens if the normal, slow decline is accelerated by chemotherapy. The antibody response to a booster immunization is blunted in some patients (the HR group in this study) after chemotherapy. Adapted from Traggiai, Puzone and Lanzavecchia (116).*

The defective memory response may be caused, at least partially, by a decrease in $CD27^+$ B cells, which are considered to confer immunological memory and are reduced after treatment for ALL (36, 117). We used enumeration of AbSC in peripheral blood as a measure of the vaccine induced B cell response. Immunizations typically lead to an increase of both the total number of AbSC and antigen specific AbSC in normal individuals (105, 115). The cellular response to immunization was increased in SR and normal in IR. In the HR group both total numbers of IgG AbSC (with unknown specificity) and TT and Hib specific IgG and IgA AbSC were low at 7 days after immunization, consistent with a defective memory response.

The origin of the poor immune response is not revealed by a quantitative analysis of B cells, since the total B cells were higher in HR than SR+IR. Considering that the frequency of total IgG AbSC and TT^+ IgG AbSC at 7 days after vaccination can be estimated to be 7/1000 and 1/1000 respectively of the total B cell number[1], it is not surprising that no correlation was found between the quantity of B cells and antibody response.

Analysis of B cell subsets showed that the number of $CD5^+$ B cells, suggested to be the human counterpart of the B1 cells in mice, is increased during reconstitution. The relative and absolute increase was most pronounced in the HR group. B1 cells are believed to be part of an intermediate early response system ("natural memory"), that together with the marginal zone B cells of the spleen, bridges the time gap between the rapid innate and the more slowly evolving adaptive immunity (118). B1 and marginal zone cells characteristically produce a rapid IgM response to blood-borne antigens in a T cell independent pathway, giving short-term protective immunity to bacterial polysaccharides. Immunological memory does not develop in the B1 subset, and the isotypes produced are mainly IgM, IgG3 and IgA (119). An expanded B1 subset after chemotherapy may at least in part explain why invasive bacterial infections are relatively uncommon after treatment, despite defects in the adaptive immune system (17). Increased B1 cells may contribute to the poor immune response in the HR group, since negative correlations were found between $CD5^+19^+$ and anti-TT IgG/anti-Hib IgG after immunization (data not shown). Further, we found increased IgG3 levels both at 1 and 6 months as an indication of increased B1 cell activity.

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[1] The proportion of lymphocytes in PBMC was 87% in our lab (mean of 10 samples; personal communication B.A. Andersson). The median value of B cells in the control group was 15% of PBMC $\Rightarrow 0.15 \times 0.87 \times 10^6 = 130 \times 10^5$ B cells / 10^6 PBMC. The total IgG AbSC were $1000/10^6$ PBMC and TT^+ IgG AbSC $200/10^6$ PBMC.

Defects in T cells

The memory response to antigens is a special property of protein antigens. Preformed memory B cells interact with cognate helper T cells and when activated differentiate to plasmablasts (113). Since chemotherapy leads to depletion of T cells, particularly long standing for $CD4^+$ subsets, defective T cell help can be anticipated (21, 25) Indeed, the total T cells, $CD4^+$, $CD4^+45RA^+$ and $CD4^+45RO^+$ subsets were decreased at +6 months, but no significant differences between IR and HR were found. T cell responses to mitogens were roughly normal at +6 months, also without differences between the risk groups. In contrast to HR, the IR group produced high levels of specific antibodies with normal avidities demonstrating that the actual size of the major peripheral T cell populations does not predict the response to immunizations. Immunization with TT causes an increase of TT-specific $CD4^+$ T cells in normal individuals, and it remains to be determined how antigen-specific T cells are affected by chemotherapy (120).

Immune reconstitution

Immune reconstitution is an important process in the restoration of the homeostasis, e g it has been convincingly shown that immune reconstitution after stem cell transplantation influences both the risk of infections and relapse of the leukemia (121). Comparing our data to previous studies of children with solid tumors or ALL confirms that recovery of $CD4^+$ T cells was not accomplished at +6 months, and shows that recovery of $CD4^+$ correlated to the $CD45RA^+$ (naive) subset also after treatment for ALL (25, 26). $CD8^+$ T cells recovered more slowly in the HR group, and at +6 months neither $CD8^+$ nor $CD8^+45RA^+$ were normalized in this study. Alanko et al found, in contrast, that $CD8^+$ T cells recovered within 3 months after ALL (27).

Vaccinations after chemotherapy

While different studies have shown that children lose immunity after chemotherapy, clinical guidelines for management have not been established (122). A few published recommendations are based on expert opinions rather than controlled studies (123, 124). It has been suggested that the immune recovery is sufficient for a good immune response to revaccinations after 3-6 months without chemotherapy (34, 123, 124). The data generated in study I-III challenge this since we noted normal antibody responses to DT, TT and Hib in SR and IR already at +1 month.

For diphtheria and tetanus subprotective antibody levels are common also in normal children in Sweden, and the unprotected proportion increases with time after immunization (125, 126). Booster immunizations are recommended to ensure long term protective immunity. Comparing our results in children with ALL to normal children shows that subprotective antibody levels are more common in patients after treatment. Information regarding the quality of immunity (primary or memory response) supplements antibody levels and can be obtained through measurement of antibody levels after vaccination, analysis of avidity or specific AbSC. In the swedish studies of normal children >95% had antibody levels above the limit for protection after booster immunization. We can also compare to our control group, of which 100% were protected after immunization. Poor immune responses were documented only in HR patients in this study.

Ara-C and the innate immune system

Fever is a frequent problem in children with cancer. Numerous causes exist, such as the malignant disorder itself, drug fever and transfusion reactions. Since infections are common and also, particularly in the setting of neutropenia, potentially life-threatening, fever must always be considered as a sign of infection (110). Ara-C is highly effective against leukemia and lymphoma, but also a good example of an anti-neoplastic agent that can cause fever in different ways. The potential to cause drug fever has been recognized for a long time, with reported incidence of 33-44% (96, 127-129). In study IV the incidence of Ara-C fever after HDAC was 67% of all courses. This higher frequency may depend on the fact that previous studies were either performed in adults or in children receiving lower doses.

Study V shows an association between HDAC and elevated plasma levels of both pro- and anti-inflammatory cytokines, suggesting that Ara-C induces a cytokine release syndrome. IL-1β, TNF-α and IL-6 are pyrogenic cytokines inducing both fever and the acute phase response, and TNF-α and IL-1β are primary inducers of inflammation often acting synergistically (130). Our data show that TNF-α levels increase before the onset of fever in all patients. Since no systemic release of IL-1β was detected, it can be speculated that TNF-α is the prime mediator, inducing the production of IL-6 and IFN-γ. However, it must be held in mind that measurements of IL-1β are more unreliable than for TNF-α and IL-6 (131). The correlation between IL-6 levels and

CRP indicates that IL-6 may cause the moderate elevation of CRP that accompanies Ara-C fever. Later during HDAC treatment the anti-inflammatory peptides IL-1ra and IL-10 increase. Since anti-inflammatory cytokines are beleived to counter-balance the inflammatory response, the increased plasma levels of IL1-ra and IL-10 may contribute to the spontaneous resolution of the Ara-C syndrome.

Procalcitonin was also shown to increase in plasma during Ara-C fever. Three of 16 non-neutropenic patients had PCT >1.5 ng/ml at 36 hours after start of the first infusion, which are levels that are normally seen in bacterial infections (132). The production of PCT in monocytes can be stimulated by IL-1β, TNF-α and IL-6 (133). A correlation between levels of CRP and PCT was found, but it can be concluded that determinations of CRP and PCT have low specificity in patients with Ara-C fever.

The molecular mechanisms behind Ara-C induced activation of the innate immune system are unknown. Ara-C can activate the transcription factor nuclear factor kappa B (NF-κB), which is a critical mediator for the upregulation of the cytokine network leading to an inflammatory host response (134, 135). NF-κB can also have anti-apoptotic effects and NF-κB inhibition is explored as a way of increasing Ara-C induced apoptosis in human acute myeloid leukemia cells (136, 137). One way of reducing NF-κB activation is through glucocorticoids, so this could explain the efficient inhibition of Ara-C fever by steroids (138).

Is cytokine release a unique property of Ara-C or a generic reaction to cytotoxic chemotherapy? Gemcitabine, a nucleoside analog derived from Ara-C, can cause fever and lung toxicity (139, 140). A study on mice showed that gemcitabine can induce the expression of proinflammatory cytokine genes in lung tissue (141). Taxanes (paclitaxel and docetaxel) can also lead to cytokine release (142, 143). However, some of the most commonly used drugs in ALL protocols (high dose methotrexate, cyclophosphamide, doxorubicin) do not cause increased plasma levels of TNF-α, IL-6 or IFN-γ (Jonas Abrahamsson, unpublished data).

Besides these activating effects, Ara-C also has strong suppressive effects on the innate immune system. Myelosuppression is a primary side effect of HDAC (81, 85). Neutropenic fever is very frequent, occuring after 40-50 % of the cycles (85, 91). We found an incidence of febrile episodes during the neutropenic phase of 55% (IV). In a study of the total incidence of infections in childhood ALL it was shown that fever was observed during 24% of all chemotherapy cycles (91).

Already in 1966 Bodey found that infections were more frequent in patients with acute leukemia when both granulocytopenia and lymphopenia were present (7). Subsequent studies have confirmed that early lymphopenia (within 5 days after cytotoxic treatment), as well as $CD4^+$ lymphopenia, are independent risk factors for febrile neutropenia after chemotherapy (144, 145). Study IV demonstrates that HDAC causes both neutropenia, a profound early lymphopenia and a high incidence of infections. The emergence of lymphopenia was so early, already during HDAC, that suppression of the bone marrow cannot be the sole reason. Instead, it must be due to either pooling of peripheral lymphocytes or direct lymphotoxic effects. Thus, since Ara-C treatment is known to activate the apoptotic machinery in normal human peripheral blood lymphocytes, a rapid elimination of lymphocytes may occur (146).

We found that neutropenic fever was more common in patients that had experienced Ara-C fever. It can be discussed if the initial cytokine release during HDAC predisposes for immunosuppression and subsequent infections through deactivation of monocytes (147, 148).

Although HDAC has been reported to be an independent risk factor for VS sepsis, such infections were only documented in 2/169 HDAC courses and none of the cases were severely ill. (92, 93). The risk of VS sepsis is modified by other factors, such as underlying disorder, incomplete remission, mucositis and antibacterial prophylaxis, but our results show that when HDAC is used as monotherapy in patients in complete remission of lymphoid malignancies VS sepsis is uncommon.

The use of prophylactic G/GM CSF after HDAC to decrease the risk of febrile neutropenia has been debated. However, very little data support that growth factors actually decrease the frequency of infections (149-151). Although our study was not designed to evaluate the effect of G/GM CSF, the data convincingly show that in this particular setting HDAC can be safely administered without the use of prophylactic CSF.

CONCLUSIONS

- Antibody levels decrease during intensive ALL treatment, leaving a majority of patients with subprotective levels against diphtheria and tetanus. Reimmunizations lead to a normal immune response in the standard and intermediate risk groups already at one month after treatment, and give full protection against diphtheria, tetanus and Hib in all patients. In contrast, the high risk patients display an attenuated immune response even at six months after treatment, with low numbers of circulating specific antibody secreting cells and low antibody levels of all isotypes. Antibody avidity after immunization was low for anti-TT, but not for anti-Hib, in the high risk group. In the high risk group, immunizations are of limited value before 6 months after treatment, and the effect of repeated immunizations should be evaluated in future studies.
- Reconstitution of the adaptive immune system after childhood ALL depends on treatment intensity and full recovery of T or B cell subsets is not accomplished at 6 months after treatment. In particular, low levels of $CD4^{+}45RA^{+}$ cells and an abnormal increase in $CD5^{+}19^{+}$ (B1) cells is present, most pronounced for the HR group.
- HDAC affects the innate immune system. A systemic inflammatory response is induced in 2/3 of all courses. The pathogenesis involves a release of proinflammatory cytokines, starting with TNFα and followed by a peak of IL6 and IFN. This is counterbalanced by anti-inflammatory cytokines (IL1-ra and IL-10). The Ara-C syndrome is self-limiting, but can be inhibited by corticosteroids.
- Myelosuppression after HDAC is profound and nearly universal, and neutropenic infections occur after half of the courses. A pronounced lymphopenia, starting within the first days after treatment, may also influence the risk of infections. However, the risk of viridans streptococcal sepsis was low and there was no mortality from infections, despite that almost all HDAC was administered without use of G/GM-CSF.

SAMMANFATTNING PÅ SVENSKA

Immunrekonstitution efter leukemi hos barn och ungdomar

Aspekter på vaccinationer och effekter av högdos cytosar på den naturliga immuniteten.

Torben Ek, leg läkare

Cytostatika behandling har lett till att minst 80% av barn med akut lymfatisk leukemi botas, men immunologiska biverkningar är vanliga och potentiellt allvarliga biverkningar. Vaccinationer mot difteri, tetanus och *Haemophilus influenzae typ B* (Hib) användes för att undersöka det adaptiva immunförsvaret i en kontrollerad studie av 31 barn som behandlats för ALL. Majoriteten av patienterna hade förlorat sitt antikroppsskydd mot difteri (83%) och tetanus (67%) efter behandlingen, medan alla hade skydd mot Hib. Immunsvaret skiljde sig markant mellan de olika riskgrupperna. Alla standard och intermediär risk patienter hade bra skydd, medan hög risk gruppen, som fått mest intensiv cytostatika behandling, hade lägst antikroppsnivåer och många var oskyddade även efter vaccinationen. Hög risk patienterna hade flera tecken på ett defekt immunologiskt minne, såsom låga antikroppsnivåer och antikroppsproducerande celler efter vaccinationen. Aviditeten, ett mått på antikropparnas funktionella bindningsstyrka, hos tetanusantikropparna var också låg i den gruppen. Effekten av vaccination 1 månad eller 6 månader efter behandlingen skiljde sig inte. Den immunologiska rekonstitutionen efter ALL undersöktes genom mätning av olika subpopulationer av lymfocyter och T och B cellsfunktionen *in vitro*. Sammanfattningsvis var immunförsvaret ej normaliserat vid 6 månader efter avslutad behandling. T cellerna var reducerade, beroende på låga antal CD4+ och CD4+45RA+ T celler. CD5+ B celler ökade under rekonstitutionsprocessen, mest markant i hög risk ALL gruppen. Resultaten motiverar en förändrad policy för vaccinationer efter ALL. Inaktiverade vacciner ger bra effekt redan 1 månad efter ALL behandlingen i standard och intermediär risk patienterna. I hög risk gruppen bör effekten av upprepade vaccinationer undersökas.

Cytosar (ara-C) är ett viktigt läkemedel för behandling av leukemi och vissa lymfom. Cytosar behandling ger ofta feber (cytosar-feber) och detta undersöktes i en grupp av 57 patienter med ALL eller lymfom, som fick behandling med högdos cytosar (totalt 169 kurer). Cytosar-feber uppträdde vid 2/3 av kurerna och åtföljdes av förhöjt CRP, och i vissa fall procalcitonin. Ett samband mellan feber och frisättning av proinflammatoriska cytokiner (TNF-α, IL-6 och IFN-γ) kan förklara patogenesen till febern. Febern var självbegränsande, men kunde även inhiberas av kortikosteroider. Myelosuppression och grav lymfopeni var allmänt förekommande efter högdos-cytosar, vilket delvis förklarar den höga incidensen (55%) av neutropen feber efteråt. Incidensen av sepsis med viridans-streptokocker var låg (1.1%) och inga dödsfall inträffade. Undersökningen visar att högdos-cytosar har kraftiga effekter på immunsystemet, vilket leder till exceptionellt hög incidens av både cytosar-feber och infektionsrelaterad feber.

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REFERENCES

1. Hjalgrim LL, Rostgaard K, Schmiegelow K, Soderhall S, Kolmannskog S, Vettenranta K, et al. Age- and sex-specific incidence of childhood leukemia by immunophenotype in the Nordic countries. J Natl Cancer Inst 2003;95(20):1539-44.
2. Kreuger A, Berglund G, Garwicz S, Mellander L, Soderhall S, Ahstrom L, et al. [Acute lymphatic leukemia in Swedish children 1968-2001. The marked improvement of the survival can be ascribed to successful treatment]. Lakartidningen 2004;101(48):3890, 3893-4, 3897-8.
3. Smith M, Arthur D, Camitta B, Carroll AJ, Crist W, Gaynon P, et al. Uniform approach to risk classification and treatment assignment for children with acute lymphoblastic leukemia. J Clin Oncol 1996;14(1):18-24.
4. Gustafsson G, Kreuger A, Clausen N, Garwicz S, Kristinsson J, Lie SO, et al. Intensified treatment of acute childhood lymphoblastic leukaemia has improved prognosis, especially in non-high-risk patients: the Nordic experience of 2648 patients diagnosed between 1981 and 1996. Nordic Society of Paediatric Haematology and Oncology (NOPHO). Acta Paediatr 1998;87(11):1151-61.
5. Saarinen-Pihkala UM, Gustafsson G, Carlsen N, Flaegstad T, Forestier E, Glomstein A, et al. Outcome of children with high-risk acute lymphoblastic leukemia (HR-ALL): Nordic results on an intensive regimen with restricted central nervous system irradiation. Pediatr Blood Cancer 2004;42(1):8-23.
6. Gustafsson G, Schmiegelow K, Forestier E, Clausen N, Glomstein A, Jonmundsson G, et al. Improving outcome through two decades in childhood ALL in the Nordic countries: the impact of high-dose methotrexate in the reduction of CNS irradiation. Nordic Society of Pediatric Haematology and Oncology (NOPHO). Leukemia 2000;14(12):2267-75.
7. Bodey GP, Buckley M, Sathe YS, Freireich EJ. Quantitative relationships between circulating leukocytes and infection in patients with acute leukemia. Ann Intern Med 1966;64(2):328-40.
8. Howell KM. The failure of antibody production in leukemia. Arch Intern Med 1920;26:706-714.
9. Ogra PL, Sinks LF, Karzon DT. Poliovirus antibody response in patients with acute leukemia. J Pediatr 1971;79(3):444-9.
10. Borella L, Webster RG. The immunosuppressive effects of long-term combination chemotherapy in children with acute leukemia in remission. Cancer Res 1971;31(4):420-6.
11. Lehrnbecher T, Foster C, Vazquez N, Mackall CL, Chanock SJ. Therapy-induced alterations in host defense in children receiving therapy for cancer. J Pediatr Hematol Oncol 1997;19(5):399-417.
12. Feldman S, Lott L. Varicella in children with cancer: impact of antiviral therapy and prophylaxis. Pediatrics 1987;80(4):465-72.
13. Hall CB, Powell KR, MacDonald NE, Gala CL, Menegus ME, Suffin SC, et al. Respiratory syncytial viral infection in children with compromised immune function. N Engl J Med 1986;315(2):77-81.
14. Gray MM, Hann IM, Glass S, Eden OB, Jones PM, Stevens RF. Mortality and morbidity caused by measles in children with malignant disease attending four major treatment centres: a retrospective review. Br Med J (Clin Res Ed) 1987;295(6589):19-22.
15. Poulsen A, Schmiegelow K, Yssing M. Varicella zoster infections in children with acute lymphoblastic leukemia. Pediatr Hematol Oncol 1996;13(3):231-8.
16. Feldman S, Gigliotti F, Shenep JL, Roberson PK, Lott L. Risk of Haemophilus influenzae type b disease in children with cancer and response of immunocompromised leukemic children to a conjugate vaccine. J Infect Dis 1990;161(5):926-31.
17. Siber GR. Bacteremias due to Haemophilus influenzae and Streptococcus pneumoniae: their occurrence and course in children with cancer. Am J Dis Child 1980;134(7):668-72.
18. Mackall CL. T-cell immunodeficiency following cytotoxic antineoplastic therapy: a review. Stem Cells 2000;18(1):10-8.
19. de Vaan GA, van Munster PJ, Bakkeren JA. Recovery of immune function after cessation of maintenance therapy in acute lymphoblastic leukemia (ALL) of childhood. Eur J Pediatr 1982;139(2):113-7.
20. de Boer AW, de Vaan GA, Weemaes CM, Bakkeren JA. Iatrogenic IgG2 deficiency in a leukaemic child. A case report. Eur J Pediatr 1992;151(4):271-3.
21. Mackall CL, Fleisher TA, Brown MR, Magrath IT, Shad AT, Horowitz ME, et al. Lymphocyte depletion during treatment with intensive chemotherapy for cancer. Blood 1994;84(7):2221-8.

22. Poulsen A, Demeny AK, Bang Plum C, Gjerum Nielsen K, Schmiegelow K. Pneumocystis carinii pneumonia during maintenance treatment of childhood acute lymphoblastic leukemia. Med Pediatr Oncol 2001;37(1):20-3.
23. Moritz B, Eder J, Meister B, Heitger A. Intact T-cell regenerative capacity in childhood acute lymphoblastic leukemia after remission induction therapy. Med Pediatr Oncol 2001;36(2):283-9.
24. Lovat PE, Robinson JH, Windebank KP, Kernahan J, Watson JG. Serial study of T lymphocytes in childhood leukemia during remission. Pediatr Hematol Oncol 1993;10(2):129-39.
25. Mackall CL, Fleisher TA, Brown MR, Andrich MP, Chen CC, Feuerstein IM, et al. Age, thymopoiesis, and CD4+ T-lymphocyte regeneration after intensive chemotherapy. N Engl J Med 1995;332(3):143-9.
26. Mackall CL, Fleisher TA, Brown MR, Andrich MP, Chen CC, Feuerstein IM, et al. Distinctions between CD8+ and CD4+ T-cell regenerative pathways result in prolonged T-cell subset imbalance after intensive chemotherapy. Blood 1997;89(10):3700-7.
27. Alanko S, Salmi TT, Pelliniemi TT. Recovery of blood T-cell subsets after chemotherapy for childhood acute lymphoblastic leukemia. Pediatr Hematol Oncol 1994;11(3):281-92.
28. Azuma E, Nagai M, Qi J, Umemoto M, Hirayama M, Kumamoto T, et al. CD4+ T-lymphocytopenia in long-term survivors following intensive chemotherapy in childhood cancers. Med Pediatr Oncol 1998;30(1):40-5.
29. Nash KA, Mohammed G, Nandapalan N, Kernahan J, Scott R, Craft AW, et al. T cell function in children with acute lymphoblastic leukaemia. Br J Haematol 1993;83(3):419-27.
30. Komada Y, Zhang SL, Zhou YW, Hanada M, Shibata T, Azuma E, et al. Cellular immunosuppression in children with acute lymphoblastic leukemia: effect of consolidation chemotherapy. Cancer Immunol Immunother 1992;35(4):271-6.
31. Alanko S, Salmi TT, Pelliniemi TT. Recovery of natural killer cells after chemotherapy for childhood acute lymphoblastic leukemia and solid tumors. Med Pediatr Oncol 1995;24(6):373-8.
32. Lowdell MW, Craston R, Samuel D, Wood ME, O'Neill E, Saha V, et al. Evidence that continued remission in patients treated for acute leukaemia is dependent upon autologous natural killer cells. Br J Haematol 2002;117(4):821-7.
33. Caver TE, Slobod KS, Flynn PM, Behm FG, Hudson MM, Turner EV, et al. Profound abnormality of the B/T lymphocyte ratio during chemotherapy for pediatric acute lymphoblastic leukemia. Leukemia 1998;12(4):619-22.
34. Alanko S, Pelliniemi TT, Salmi TT. Recovery of blood B-lymphocytes and serum immunoglobulins after chemotherapy for childhood acute lymphoblastic leukemia. Cancer 1992;69(6):1481-6.
35. Abrahamsson J, Marky I, Mellander L. Immunoglobulin levels and lymphocyte response to mitogenic stimulation in children with malignant disease during treatment and follow-up. Acta Paediatr 1995;84(2):177-82.
36. Nilsson A. Regulation of antibody production in immunocompromised patients. Stockholm: Karolinska Institutet; 2004.
37. Nilsson A, De Milito A, Engstrom P, Nordin M, Narita M, Grillner L, et al. Current chemotherapy protocols for childhood acute lymphoblastic leukemia induce loss of humoral immunity to viral vaccination antigens. Pediatrics 2002;109(6):e91.
38. Smith S, Schiffman G, Karayalcin G, Bonagura V. Immunodeficiency in long-term survivors of acute lymphoblastic leukemia treated with Berlin-Frankfurt-Munster therapy. J Pediatr 1995;127(1):68-75.
39. Ahmed R, Gray D. Immunological memory and protective immunity: understanding their relation. Science 1996;272(5258):54-60.
40. McHeyzer-Williams MG, Ahmed R. B cell memory and the long-lived plasma cell. Curr Opin Immunol 1999;11(2):172-9.
41. Hammarlund E, Lewis MW, Hansen SG, Strelow LI, Nelson JA, Sexton GJ, et al. Duration of antiviral immunity after smallpox vaccination. Nat Med 2003;9(9):1131-7.
42. Crotty S, Felgner P, Davies H, Glidewell J, Villarreal L, Ahmed R. Cutting edge: long-term B cell memory in humans after smallpox vaccination. J Immunol 2003;171(10):4969-73.
43. Edwards KM. Development, acceptance, and use of immunologic correlates of protection in monitoring the effectiveness of combination vaccines. Clin Infect Dis 2001;33 Suppl 4:S274-7.
44. Melville-Smith M, Balfour A. Estimation of Corynebacterium diphtheriae antitoxin in human sera: a comparison of an enzyme-linked immunosorbent assay with the toxin neutralisation test. J Med Microbiol 1988;25(4):279-83.

45. Walory J, Grzesiowski P, Hryniewicz W. Comparison of four serological methods for the detection of diphtheria anti-toxin antibody. J Immunol Methods 2000;245(1-2):55-65.
46. Gupta RK, Siber GR. Comparative analysis of tetanus antitoxin titers of sera from immunized mice and guinea pigs determined by toxin neutralization test and enzyme-linked immunosorbent assay. Biologicals 1994;22(3):215-9.
47. Carlsson R. Evaluation of vaccines in infancy. With special reference to Haemophilus influenzae type B conjugates: Goteborg University; 1999.
48. Steward MW, Lew AM. The importance of antibody affinity in the performance of immunoassays for antibody. J Immunol Methods 1985;78(2):173-90.
49. Plotkin SA. Immunologic correlates of protection induced by vaccination. Pediatr Infect Dis J 2001;20(1):63-75.
50. Galazka AM. The immunological basis for immunization series (3): Tetanus. Expanded programme on immunization, WHO Geneva 1993; 1993.
51. Galazka AM. The immunological basis for immunization series (2): Diphtheria. Expanded programme on immunization, WHO Geneva 1993. 1993.
52. Kayhty H, Peltola H, Karanko V, Makela PH. The protective level of serum antibodies to the capsular polysaccharide of Haemophilus influenzae type b. J Infect Dis 1983;147(6):1100.
53. Macdonald RA, Hosking CS, Jones CL. The measurement of relative antibody affinity by ELISA using thiocyanate elution. J Immunol Methods 1988;106(2):191-4.
54. Roberton DM, Carlsson B, Coffman K, Hahn-Zoric M, Jalil F, Jones C, et al. Avidity of IgA antibody to Escherichia coli polysaccharide and diphtheria toxin in breast milk from Swedish and Pakistani mothers. Scand J Immunol 1988;28(6):783-9.
55. Bachmann MF, Kalinke U, Althage A, Freer G, Burkhart C, Roost H, et al. The role of antibody concentration and avidity in antiviral protection. Science 1997;276(5321):2024-7.
56. Lazzarotto T, Spezzacatena P, Pradelli P, Abate DA, Varani S, Landini MP. Avidity of immunoglobulin G directed against human cytomegalovirus during primary and secondary infections in immunocompetent and immunocompromised subjects. Clin Diagn Lab Immunol 1997;4(4):469-73.
57. Goldblatt D, Vaz AR, Miller E. Antibody avidity as a surrogate marker of successful priming by Haemophilus influenzae type b conjugate vaccines following infant immunization. J Infect Dis 1998;177(4):1112-5.
58. Abrahamsson J, Carlsson B, Mellander L. Serum antibody levels and avidities to Escherichia coli O antigens and poliovirus type 1 antigen are increased in children treated for malignant disease. Med Pediatr Oncol 1999;32(6):416-20.
59. Rajouria VP, Johnston JH. Tetanus complicating acute leukaemia--a case report. Ann Trop Med Parasitol 1982;76(2):247.
60. Dittmann S, Wharton M, Vitek C, Ciotti M, Galazka A, Guichard S, et al. Successful control of epidemic diphtheria in the states of the Former Union of Soviet Socialist Republics: lessons learned. J Infect Dis 2000;181 Suppl 1:S10-22.
61. Bjorkholm B, Olling S, Larsson P, Hagberg L. An outbreak of diphtheria among Swedish alcoholics. Infection 1987;15(5):354-8.
62. Romanus V. Haemophilus influenzae typ b (Hib) – Kommentarer till statistiken. 2003 [cited; Available from: http://www.smittskyddsinstitutet.se/SMItemplates/
63. Heath PT, Booy R, Griffiths H, Clutterbuck E, Azzopardi HJ, Slack MP, et al. Clinical and immunological risk factors associated with Haemophilus influenzae type b conjugate vaccine failure in childhood. Clin Infect Dis 2000;31(4):973-80.
64. Rowland P, Wald ER, Mirro JR, Jr., Yunis E, Albo VC, Wollman MR, et al. Progressive varicella presenting with pain and minimal skin involvement in children with acute lymphoblastic leukemia. J Clin Oncol 1995;13(7):1697-703.
65. McCusker C, Somerville W, Grey V, Mazer B. Specific antibody responses to diphtheria/tetanus revaccination in children evaluated for immunodeficiency. Ann Allergy Asthma Immunol 1997;79(2):145-50.
66. Fleisher TA, Oliveira JB. Functional and molecular evaluation of lymphocytes. J Allergy Clin Immunol 2004;114(2):227-34; quiz 235.
67. van der Does-van den Berg A, Hermans J, Nagel J, van Steenis G. Immunity to diphtheria, pertussis, tetanus, and poliomyelitis in children with acute lymphocytic leukemia after cessation of chemotherapy. Pediatrics 1981;67(2):222-9.
68. Kung FH, Orgel HA, Wallace WW, Hamburger RN. Antibody production following immunization with diphtheria and tetanus toxoids in children receiving chemotherapy during remission of malignant disease. Pediatrics 1984;74(1):86-9.
69. Ridgway D, Wolff LJ, Deforest A. Immunization response varies with intensity of acute lymphoblastic leukemia therapy. Am J Dis Child 1991;145(8):887-91.

70. Hovi L, Valle M, Siimes MA, Jalanko H, Saarinen UM. Impaired response to hepatitis B vaccine in children receiving anticancer chemotherapy. Pediatr Infect Dis J 1995;14(11):931-5.
71. Mustafa MM, Buchanan GR, Winick NJ, McCracken GH, Tkaczewski I, Lipscomb M, et al. Immune recovery in children with malignancy after cessation of chemotherapy. J Pediatr Hematol Oncol 1998;20(5):451-7.
72. von der Hardt K, Jungert J, Beck JD, Heininger U. Humoral immunity against diphtheria, tetanus and poliomyelitis after antineoplastic therapy in children and adolescents--a retrospective analysis. Vaccine 2000;18(26):2999-3004.
73. Reinhardt D, Houliara K, Pekrun A, Lakomek M, Krone B. Impact of conventional chemotherapy on levels of antibodies against vaccine-preventable diseases in children treated for cancer. Scand J Infect Dis 2003;35(11-12):851-7.
74. Zignol M, Peracchi M, Tridello G, Pillon M, Fregonese F, D'Elia R, et al. Assessment of humoral immunity to poliomyelitis, tetanus, hepatitis B, measles, rubella, and mumps in children after chemotherapy. Cancer 2004;101(3):635-41.
75. Fioredda F, Plebani A, Hanau G, Haupt R, Giacchino M, Barisone E, et al. Re-immunisation schedule in leukaemic children after intensive chemotherapy: a possible strategy. Eur J Haematol 2005;74(1):20-3.
76. Ridgway D, Wolff LJ. Active immunization of children with leukemia and other malignancies. Leuk Lymphoma 1993;9(3):177-92.
77. Ek T, Mellander L, Hahn-Zoric M, Abrahamsson J. Intensive Treatment for Childhood Acute Lymphoblastic Leukemia Reduces Immune Responses to Diphtheria, Tetanus, and Haemophilus influenzae Type b. J Pediatr Hematol Oncol 2004;26(11):727-734.
78. Kantar M, Cetingul N, Kansoy S, Kutukculer N, Aksu G. Immune deficiencies following cancer treatment in children. J Trop Pediatr 2003;49(5):286-90.
79. Lange B, Jakacki R, Nasab AH, Luery N, McVerry PH. Immunization of leukemic children with Haemophilus conjugate vaccine. Pediatr Infect Dis J 1989;8(12):883-4.
80. Weisman SJ, Cates KL, Allegretta GJ, Quinn JJ, Altman AJ. Antibody response to immunization with Haemophilus influenzae type b polysaccharide vaccine in children with cancer. J Pediatr 1987;111(5):727-9.
81. Chabner B. Cytidine analogues. In: Chabner B, Longo D, editors. Cancer Chemotherapy and Biotherapy. 2nd ed. Philadelphia: JB Lippincott; 1996. p. 213-233.
82. Estlin EJ, Yule SM, Lowis SP. Consolidation therapy for childhood acute lymphoblastic leukaemia: clinical and cellular pharmacology of cytosine arabinoside, epipodophyllotoxins and cyclophosphamide. Cancer Treat Rev 2001;27(6):339-50.
83. Reiter A, Schrappe M, Ludwig WD, Hiddemann W, Sauter S, Henze G, et al. Chemotherapy in 998 unselected childhood acute lymphoblastic leukemia patients. Results and conclusions of the multicenter trial ALL-BFM 86. Blood 1994;84(9):3122-33.
84. Harris RE, Sather HN, Feig SA. High-dose cytosine arabinoside and L-asparaginase in refractory acute lymphoblastic leukemia: the Children's Cancer Group experience. Med Pediatr Oncol 1998;30(4):233-9.
85. Barrios NJ, Tebbi CK, Freeman AI, Brecher ML. Toxicity of high dose Ara-C in children and adolescents. Cancer 1987;60(2):165-9.
86. Gottlieb D, Bradstock K, Koutts J, Robertson T, Lee C, Castaldi P. The neurotoxicity of high-dose cytosine arabinoside is age-related. Cancer 1987;60(7):1439-41.
87. Jehn U, Goldel N, Rienmuller R, Wilmanns W. Non-cardiogenic pulmonary edema complicating intermediate and high-dose Ara C treatment for relapsed acute leukemia. Med Oncol Tumor Pharmacother 1988;5(1):41-7.
88. Andersson BS, Luna MA, Yee C, Hui KK, Keating MJ, McCredie KB. Fatal pulmonary failure complicating high-dose cytosine arabinoside therapy in acute leukemia. Cancer 1990;65(5):1079-84.
89. Shearer P, Katz J, Bozeman P, Jenkins J, Laver J, Krance R, et al. Pulmonary insufficiency complicating therapy with high dose cytosine arabinoside in five pediatric patients with relapsed acute myelogenous leukemia. Cancer 1994;74(7):1953-8.
90. Chiche D, Pico JL, Bernaudin JF, Chouaib S, Wollman E, Arnoux A, et al. Pulmonary edema and shock after high-dose aracytine-C for lymphoma; possible role of TNF-alpha and PAF. Eur Cytokine Netw 1993;4(2):147-51.
91. Lex C, Korholz D, Kohlmuller B, Bonig H, Willers R, Kramm CM, et al. Infectious complications in children with acute lymphoblastic leukemia and T-cell lymphoma--a rationale for tailored supportive care. Support Care Cancer 2001;9(7):514-21.
92. Bochud PY, Eggiman P, Calandra T, Van Melle G, Saghafi L, Francioli P. Bacteremia due to viridans streptococcus in neutropenic patients with cancer: clinical spectrum and risk factors. Clin Infect Dis 1994;18(1):25-31.

93. Paganini H, Staffolani V, Zubizarreta P, Casimir L, Lopardo H, Luppino V. Viridans streptococci bacteraemia in children with fever and neutropenia: a case-control study of predisposing factors. Eur J Cancer 2003;39(9):1284-9.
94. Shenep JL. Viridans-group streptococcal infections in immunocompromised hosts. Int J Antimicrob Agents 2000;14(2):129-35.
95. Goldstein B, Giroir B, Randolph A. International pediatric sepsis consensus conference: definitions for sepsis and organ dysfunction in pediatrics. Pediatr Crit Care Med 2005;6(1):2-8.
96. Castleberry RP, Crist WM, Holbrook T, Malluh A, Gaddy D. The cytosine arabinoside (Ara-C) syndrome. Med Pediatr Oncol 1981;9(3):257-64.
97. Tarkowski A, Lue C, Moldoveanu Z, Kiyono H, McGhee JR, Mestecky J. Immunization of humans with polysaccharide vaccines induces systemic, predominantly polymeric IgA2-subclass antibody responses. J Immunol 1990;144(10):3770-8.
98. Gendrel D, Bohuon C. Procalcitonin as a marker of bacterial infection. Pediatr Infect Dis J 2000;19(8):679-87; quiz 688.
99. Meisner M. Procalcitonin (PCT). 3rd ed. Rottenburg: Georg Thieme Verlag; 2000.
100. Bernard L, Ferriere F, Casassus P, Malas F, Leveque S, Guillevin L, et al. Procalcitonin as an early marker of bacterial infection in severely neutropenic febrile adults. Clin Infect Dis 1998;27(4):914-5.
101. Fleischhack G, Kambeck I, Cipic D, Hasan C, Bode U. Procalcitonin in paediatric cancer patients: its diagnostic relevance is superior to that of C-reactive protein, interleukin 6, interleukin 8, soluble interleukin 2 receptor and soluble tumour necrosis factor receptor II. Br J Haematol 2000;111(4):1093-102.
102. Pullen GR, Fitzgerald MG, Hosking CS. Antibody avidity determination by ELISA using thiocyanate elution. J Immunol Methods 1986;86(1):83-7.
103. Qvarfordt I, Riise GC, Larsson S, Almqvist G, Rollof J, Bengtsson T, et al. Immunological findings in blood and bronchoalveolar lavage fluid in chronic bronchitis patients with recurrent infectious exacerbations. Eur Respir J 1998;11(1):46-54.
104. Leyendeckers H, Odendahl M, Lohndorf A, Irsch J, Spangfort M, Miltenyi S, et al. Correlation analysis between frequencies of circulating antigen-specific IgG-bearing memory B cells and serum titers of antigen-specific IgG. Eur J Immunol 1999;29(4):1406-17.
105. Odendahl M, Mei H, Hoyer BF, Jacobi AM, Hansen A, Muehlinghaus G, et al. Generation of migratory antigen-specific plasma blasts and mobilization of resident plasma cells in a secondary immune response. Blood 2005;105(4):1614-21.
106. Czerkinsky CC, Nilsson LA, Nygren H, Ouchterlony O, Tarkowski A. A solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody-secreting cells. J Immunol Methods 1983;65(1-2):109-21.
107. Altman D. Practical statistics for medical research: Chapm & Hall/CRC; 1999.
108. Von Hoff DD, Layard MW, Basa P, Davis HL, Jr., Von Hoff AL, Rozencweig M, et al. Risk factors for doxorubicin-induced congestive heart failure. Ann Intern Med 1979;91(5):710-7.
109. Loebstein R, Atanackovic G, Bishai R, Wolpin J, Khattak S, Hashemi G, et al. Risk factors for long-term outcome of ifosfamide-induced nephrotoxicity in children. J Clin Pharmacol 1999;39(5):454-61.
110. Pizzo PA, Poplack DG. Principles and Practice of Pediatric Oncology. Third ed. Philadelphia: Lippincott - Raven; 1997.
111. Lutsiak C, Semnani RT, De Pascalis R, Kashmiri SV, Schlom J, Sabzevari H. Inhibition of CD4+25+ T regulatory cell function implicated in enhanced immune response by low dose cyclophosphamide. Blood 2004.
112. Abrahamsson J, Mellander L. Bone marrow immunoglobulin-secreting cells are not reduced in children with leukaemia as compared to children with solid tumours. Acta Paediatr 1997;86(2):165-9.
113. McHeyzer-Williams MG. B cells as effectors. Curr Opin Immunol 2003;15(3):354-61.
114. Slifka MK, Antia R, Whitmire JK, Ahmed R. Humoral immunity due to long-lived plasma cells. Immunity 1998;8(3):363-72.
115. Bernasconi NL, Traggiai E, Lanzavecchia A. Maintenance of serological memory by polyclonal activation of human memory B cells. Science 2002;298(5601):2199-202.
116. Traggiai E, Puzone R, Lanzavecchia A. Antigen dependent and independent mechanisms that sustain serum antibody levels. Vaccine 2003;21 Suppl 2:S35-7.
117. Agematsu K, Hokibara S, Nagumo H, Komiyama A. CD27: a memory B-cell marker. Immunol Today 2000;21(5):204-6.
118. Martin F, Oliver AM, Kearney JF. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. Immunity 2001;14(5):617-29.

119. Martin F, Kearney JF. B1 cells: similarities and differences with other B cell subsets. Curr Opin Immunol 2001;13(2):195-201.
120. Mayer S, Laumer M, Mackensen A, Andreesen R, Krause SW. Analysis of the immune response against tetanus toxoid: enumeration of specific T helper cells by the Elispot assay. Immunobiology 2002;205(3):282-9.
121. Auletta JJ, Lazarus HM. Immune restoration following hematopoietic stem cell transplantation: an evolving target. Bone Marrow Transplant 2005.
122. Mahajan A, English MW, Jenney ME, Foot A. Survey of immunisation practices in the United Kingdom during and following completion of anti-cancer chemotherapy in children. Med Pediatr Oncol 2003;40(4):270-1.
123. IMMUNISATION OF THE IMMUNOCOMPROMISED CHILD Best Practice Statement. 2002 Available from: http://www.rcpch.ac.uk/publications/recent_publications/Immunocomp.pdf
124. Graubner UB, Liese J, Belohradsky BH. [Vaccination]. Klin Padiatr 2001;213 Suppl 1:A77-83.
125. Christenson B, Bottiger M. Immunity and immunization of children against tetanus in sweden. Scand J Infect Dis 1991;23(5):643-7.
126. Mark A, Christenson B, Granstrom M, Strandell A, Wickbom B, Bottiger M. Immunity and immunization of children against diphtheria in Sweden. Eur J Clin Microbiol Infect Dis 1989;8(3):214-9.
127. Rose MS, Bateman DN. Pyrexia with cytosine arabinoside. Br Med J 1972;4(832):115.
128. Manoharan A. The cytarabine syndrome in adults. Aust N Z J Med 1985;15(4):451-2.
129. Gonen C, Celik I, Cetinkaya YS, Haznedaroglu I. Cytarabine-induced fever complicating the clinical course of leukemia. Anticancer Drugs 2005;16(1):59-62.
130. Dinarello CA. Proinflammatory cytokines. Chest 2000;118(2):503-8.
131. Dinarello CA. Cytokines as endogenous pyrogens. J Infect Dis 1999;179 Suppl 2:S294-304.
132. Gendrel D, Raymond J, Coste J, Moulin F, Lorrot M, Guerin S, et al. Comparison of procalcitonin with C-reactive protein, interleukin 6 and interferon-alpha for differentiation of bacterial vs. viral infections. Pediatr Infect Dis J 1999;18(10):875-81.
133. Oberhoffer M, Stonans I, Russwurm S, Stonane E, Vogelsang H, Junker U, et al. Procalcitonin expression in human peripheral blood mononuclear cells and its modulation by lipopolysaccharides and sepsis-related cytokines in vitro. J Lab Clin Med 1999;134(1):49-55.
134. Yamamoto Y, Gaynor RB. Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer. J Clin Invest 2001;107(2):135-42.
135. Brach MA, Kharbanda SM, Herrmann F, Kufe DW. Activation of the transcription factor kappa B in human KG-1 myeloid leukemia cells treated with 1-beta-D-arabinofuranosylcytosine. Mol Pharmacol 1992;41(1):60-3.
136. Wang CY, Mayo MW, Baldwin AS, Jr. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. Science 1996;274(5288):784-7.
137. Frelin C, Imbert V, Griessinger E, Peyron AC, Rochet N, Philip P, et al. Targeting NF-kappaB activation via pharmacologic inhibition of IKK2-induced apoptosis of human acute myeloid leukemia cells. Blood 2005;105(2):804-11.
138. Scheinman RI, Cogswell PC, Lofquist AK, Baldwin AS, Jr. Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids. Science 1995;270(5234):283-6.
139. Aapro MS, Martin C, Hatty S. Gemcitabine--a safety review. Anticancer Drugs 1998;9(3):191-201.
140. Barlesi F, Villani P, Doddoli C, Gimenez C, Kleisbauer JP. Gemcitabine-induced severe pulmonary toxicity. Fundam Clin Pharmacol 2004;18(1):85-91.
141. Rube CE, Wilfert F, Uthe D, Konig J, Liu L, Schuck A, et al. Increased expression of pro-inflammatory cytokines as a cause of lung toxicity after combined treatment with gemcitabine and thoracic irradiation. Radiother Oncol 2004;72(2):231-41.
142. Tsavaris N, Kosmas C, Vadiaka M, Kanelopoulos P, Boulamatsis D. Immune changes in patients with advanced breast cancer undergoing chemotherapy with taxanes. Br J Cancer 2002;87(1):21-7.
143. Pusztai L, Mendoza TR, Reuben JM, Martinez MM, Willey JS, Lara J, et al. Changes in plasma levels of inflammatory cytokines in response to paclitaxel chemotherapy. Cytokine 2004;25(3):94-102.
144. Blay JY, Chauvin F, Le Cesne A, Anglaret B, Bouhour D, Lasset C, et al. Early lymphopenia after cytotoxic chemotherapy as a risk factor for febrile neutropenia. J Clin Oncol 1996;14(2):636-43.

145. Borg C, Ray-Coquard I, Philip I, Clapisson G, Bendriss-Vermare N, Menetrier-Caux C, et al. CD4 lymphopenia as a risk factor for febrile neutropenia and early death after cytotoxic chemotherapy in adult patients with cancer. Cancer 2004;101(11):2675-2680.
146. Stahnke K, Fulda S, Friesen C, Strauss G, Debatin KM. Activation of apoptosis pathways in peripheral blood lymphocytes by in vivo chemotherapy. Blood 2001;98(10):3066-73.
147. Docke WD, Randow F, Syrbe U, Krausch D, Asadullah K, Reinke P, et al. Monocyte deactivation in septic patients: restoration by IFN-gamma treatment. Nat Med 1997;3(6):678-81.
148. Volk HD, Reinke P, Krausch D, Zuckermann H, Asadullah K, Muller JM, et al. Monocyte deactivation--rationale for a new therapeutic strategy in sepsis. Intensive Care Med 1996;22 Suppl 4:S474-81.
149. Chen SH, Liang DC, Liu HC. High-dose cytarabine-containing chemotherapy with or without granulocyte colony-stimulating factor for children with acute leukemia. Am J Hematol 1998;58(1):20-3.
150. Saarinen-Pihkala UM, Lanning M, Perkkio M, Makipernaa A, Salmi TT, Hovi L, et al. Granulocyte-macrophage colony-stimulating factor support in therapy of high-risk acute lymphoblastic leukemia in children. Med Pediatr Oncol 2000;34(5):319-27.
151. Hofmann WK, Seipelt G, Langenhan S, Reutzel R, Schott D, Schoeffski O, et al. Prospective randomized trial to evaluate two delayed granulocyte colony stimulating factor administration schedules after high-dose cytarabine therapy in adult patients with acute lymphoblastic leukemia. Ann Hematol 2002;81(10):570-4.

APPENDIX (ARTICLE I-V)

GÖTEBORGS UNIVERSITET
GÖTEBORGS UNIVERSITETSBIBLIOTEK

Vasastadens
Bokbinderi AB

Bokbinderi & Tryckeri

Tel: 031 - 29 20 45 • Mail: info@vasastadensbokbinderi.se