Original Articles

Immunological Profiles of Patients with Chronic Myeloid Leukaemia. I. State before the Start of Treatment

(CML leukaemia / immunological profile / CRP / IL-6)

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Abstract. In view of the increasing interest in the immunotherapy of CML it seems highly desirable to broaden the present knowledge on the immune reactivity of CML patients. A group of 24 patients and 24 healthy controls were studied for the total of 15 immunological parameters, including the prevalence of antibodies against human herpesviruses and papillomaviruses. To clearly discriminate between changes associated with the disease and those induced by the therapy, all patients were enrolled prior to the start of any anti-leukaemic therapy. Statistically significant differences between patients and controls were found in the levels of IgA, C4 component of complement, CRP and IL-6, the production of Th1 cytokines in stimulated CD3 cells and the *E. coli* stimulatory index. The analysis of the interrelationship between the results obtained in the individual patients presented some unexpected findings, such as the lack of correlation between the CRP and IL-6 levels. It will be the purpose of a follow-up to determine whether and how the immune status of the patients prior to the treatment correlates with their response to therapy and how the individual immunological profiles change in the course of the disease. These observations will be utilized in the future immunotherapeutic studies to constitute the vaccine- and placebo-treated groups.

Over the past two decades a number of immunological alterations have been demonstrated in chronic myeloid leukaemia (CML) patients. Their cell-mediated immunity is frequently impaired, especially in the advanced stages of the disease (Cramer et al., 1977; Pawelec et al., 1996, 1999; Pierson and Miller, 1997; Ghalaut et al., 1999; Chen et al., 2000; Cho et al., 2000; Mellqvist et al., 2000; Reuben et al., 2000; Tsuda and Yamasaki, 2000; Nakajima et al., 2002; Chiorean et al., 2003; Kiani et al., 2003). Changes in humoral immunity have also been reported (Minh et al., 1983; Solanki et al., 1990; DuVillard et al., 1995; Kyriakou et al., 1997; Anand et al., 1998; Guc et al., 2000; Le Coutre et al., 2002). The imbalances of some of these immunological parameters have been seen to improve after

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Abbreviations: CML – chronic myeloid leukaemia, CMV – cytomegalovirus, CRP – C-reactive protein, EBV – Epstein-Barr virus, IFN – interferon, Ig – immunoglobulin, IL – interleukin, IM – imanitib mesylate, SI – stimulatory index, TNF – tumour necrosis factor, WBC – white blood cells.

Humlova.qxp 4.8.2006 10:03 Stránka 48

treatment. It may also be of interest that in some patients undergoing basophilic crisis histamine excess symptoms develop (Ishii et al., 1995). Thus, in CML patients the alterations of immune reactivity are frequent and multiform.

Changes in various immunological parameters have also been reported to be associated with therapy. Interferon-α (IFN-α), widely used in the therapy of CML, may elicit autoimmune disorders (Dacey and Callen, 2003; Steegmann et al., 2003a; Burchert and Neubauer, 2005). There is also evidence that imatinib mesylate (IM), presently the most potent anti-CML drug, exhibits, in addition to its cytostatic effects, some immunomodulating activity. Its administration has resulted in hypogammaglobulinaemia (comprising immunoglobulin (IgG, IgM and IgA) in patients previously exposed to IFN-α, and in a relative increase in the number of B lymphocytes (Steegmann et al., 2003b). It has recently also been reported that IM inhibits T-cell proliferation (Dietz et al., 2004) and dendritic cell function, at least *in vitro* (Appel et al., 2004).

In the previous immunological studies, CML patients in whom the therapy had already been started and who were in different stages of development of the disease were nearly always included. In those studies untreated and treated patients were frequently combined into a single study group. It was therefore difficult to differentiate between immune reactivities associated with the disease itself or its progression, and those induced by therapy, which may have immunosuppressive but also immunostimulatory effects. The duration, quality and significance of these alterations and their interrelationship are not fully understood. Our present investigation undertook to determine the immunological profiles of patients at diagnosis, i.e. before the initiation of any anti-leukaemic therapy. The patients will be followed prospectively, in order to find out whether there exists a correlation between their immunological profiles and their response to therapy, and how the mode and efficacy of therapy influences their immunological reactivity. As far as we are aware, no prospective follow-up study of this type has been performed as yet.

The strongest stimulus for the present undertaking has been the growing hope that therapeutic vaccines will be available for the treatment of CML in a not very distant future. We hope that the knowledge obtained will provide a more solid basis for the vaccine studies, permitting, e.g., more rigorous constitution of the groups to be vaccinated and placebo-treated and defining immune reactivities that might signalize success or failure of the vaccination.

Material and Methods

Study population

Twenty-four CML patients (9 males and 15 females), with the median age 44, range 24 to 72 years, and 24 normal healthy volunteers matched by sex and age were enrolled over the period of the last three years. Before sampling from all patients and controls (blood donors and volunteering healthy members of the laboratory personnel), written Informed Consent had been obtained and the study was approved by the Ethics Committees of all the institutions concerned. Thus, in addition to samples for routine haematological, biochemical, cytogenetic and molecular testing, material for immunological assays was obtained from each subject. The blood samples were distributed into tubes purchased from BD Vacutainer Systems, Belliver Industrial Estate, Plymouth, UK, as follows: (i) 7 ml of blood were taken into Z tubes, coagulated, and the serum was used for humoral immunity tests; (ii) 2 ml of blood were mixed with EDTA for immunophenotypic analysis; (iii) 2 ml of the whole blood were mixed with sodium heparin for measurement of intracellular cytokines; (iv) 2 ml of whole blood were mixed with lithium heparin for investigation of phagocytosis. The material was tested (see below) immediately after arrival at the laboratory. Portions of it were preserved for further tests. Sera were stored at –20°C and leukocyte suspensions were stored in liquid nitrogen.

Cytogenetic investigation

Conventional G-banded chromosomal analyses were carried out on bone marrow samples cultivated for 24 hours in RPMI 1640 medium supplemented with 10% foetal calf serum (GIBCO®, Invitrogen Corporation, Carlsbad, CA), without mitogen stimulation. Chromosomal preparations were made by standard techniques using colcemid, 0.075 M KCl as hypotonic treatment, fixation in methanol/acetic acid and G-banding with Wright's stain. Karyotypes were described according to ISCN (Mitelman, 1995). At least 22 mitoses were analysed whenever possible.

Detection of bcr–abl presence

Counts of 10^7 of isolated peripheral blood leukocytes were lysed in 0.5 ml of guanidine thiocyanate solution (Chomczynski and Sacchi, 1987) and stored at –20°C. Total RNA was isolated by the acid phenol method (Chomczynski and Sacchi, 1987) as modified by Haškovec et al. (1998) and stored at –80°C. For single-round PCR amplification of the *bcr-abl* gene and the control *bcr* gene we used the method of multiplex PCR described by Cross et al. (1994).

Immunoglobulins

The levels of total IgG, of the IgG subclasses, IgA and IgM were measured by nephelometry (Jolliff et al., 1982) using a Dade-Behring BNII™ nephelometer (Dade-Behring, Liederbach, Germany). The reagents used were as follows: OSAS15 N Antiserum to Human IgG, OQXI09 N AS IgG_1, OQXK09 N AS IgG_2, OQXL09 N AS IgG_3, OQXM09 N AS IgG_4, OSAR15

Humlova.qxp 4.8.2006 10:03 Stránka 49

N Antiserum to Human IgA and OSAT15 N Antiserum to Human IgM (Dade-Behring). The standard laboratory referential ranges are 6.90–14.0 g/l for IgG, 4.90–11.4 g/l for IgG_1, 1.50–6.40 g/l for IgG_2, 0.20–1.10 g/l for IgG_3, 0.08–1.40 g/l for IgG_4, 0.70–3.70 g/l for IgA and 0.34–2.40 g/l for IgM.

Complement

The levels of the C3 and C4 components of complement were measured by nephelometry (Jolliff et al., 1982) using a Dade-Behring BNII™ nephelometer. The reagents used were OSAP15 N Antiserum to Human C3 and OSAO15 N Antiserum to Human C4 (Dade-Behring). The standard laboratory referential ranges are 0.75–1.40 g/l for C3 and 0.10–0.34 g/l for C4.

C-reactive protein

The levels of the C-reactive protein (CRP) were measured by nephelometry (Roberts et al., 2000) using a Dade-Behring BNII™ nephelometer. The reagent used was OQIY21 N High Sensitive CRP (Dade-Behring). The standard laboratory referential range is 0.00–5.00 mg/l.

Interleukin-6

To determine the quantity of human interleukin-6 (IL-6) the Quantikine® Human IL-6 (R&D Systems, Inc., Minneapolis, MN) assay system was used. This assay utilizes the quantitative sandwich enzyme immunoassay technique. The referential range of measurement is 3.13–12.5 μg/l.

Autoantibodies

Antibodies against thyroidal peroxidase (TPO) and thyreoglobulin (TG) were measured by an AxSym (Abbott, IL) analyser unit. The standard laboratory ranges are 0–12 IU/ml for TPO and 0–34 IU/ml for TG. Antinuclear (ANAb) and antimitochondrial (AMAb) antibodies and antibodies against smooth muscles (SMAb) were detected by indirect immunoflurescence microscopy using tissue sections of rat liver, kidney and stomach purchased from The Binding Site Ltd., Birmingham, UK. Antibodies against the cytoplasm of neutrophils (ANCAb) were detected by indirect immunofluorescence microscopy using Human Granulocyte ANCA kit (ethanol-fixed; The Binding Site Ltd.). Antibodies of the IgG and IgA classes (endo G, endo A) against endomysium were tested by indirect immunofluorescence microscopy, using tissue sections from monkey oesophagus (Monkey Oesophagus (skin antibodies) IFA Kit; The Binding Site Ltd.). In all instances, the manufacturer's instructions were followed.

Subpopulations of lymphocytes

Immunophenotypic analysis of lymphocytes was performed using the flow cytometry technique and monoclonal antibodies directed against the following human surface antigens: CD3, CD4, CD8, CD19, CD16 and CD56. All monoclonal antibodies were purchased from Becton Dickinson Biosciences, BD Immunocytometry Systems, San Jose, CA. The specific clones for surface antigens were SK7 for CD3, SK3 for CD4, SK1 for CD8, SJ25C1 for CD19, B73.1 for CD16 and My31 for CD56. The tests were performed as recommended by the manufacturer. Whole leukocyte populations were exposed to the antibodies for 15 min, in the dark, at room temperature, using saturating amounts of the fluorochrome-conjugated mouse anti-human monoclonal antibody (Becton Dickinson Biosciences). Flow cytometry was performed in a FACS Calibur Becton Dickinson apparatus after lysis of red blood cells. CellQuest software (Becton Dickinson) was used for data acquisition. For the calculations, the sum of cells stained with CD4, CD8, CD19 and CD16-CD56 antibodies was considered 100%. The referential ranges are: 59–84% for $CD3^+$, 25–59% for $CD4^+$, 19–48% for $CD8^+$, 6–22% for $CD19^+$, 6–30% for $CD16^+$, $CD56^+$ cells.

Intracellular cytokine production by stimulated CD3+ cells

Intracellular cytokines IL-2, IL-4, tumour necrosis factor (TNF)-α and IFN-γ were detected by flow cytometry using a FACS Calibur Becton Dickinson apparatus. Whole blood was mixed with sodium heparin in tubes (see above), stimulated by the mixture of brefeldinA (10 μg/ml), phorbol-12-myristate-13-acetate (1 μg/ml) (all Sigma-Aldrich, St Louis, MO) and processed according to the standard protocol for assessment of intracellular cytokines in stimulated $CD3^+$ cells (Marečková et al., 2002). The following antibodies were used: Anti-Hu-IL-2 FITC (clone 5344.111), Anti-Hu-IL-4 PE (clone 3010.11), Anti-Hu-IFNγ FITC (clone 25723.11) and Anti-Hu-TNFα PE (clone 6401.1111). The figures shown in the Results section indicate the percentages of $CD3^+$ lymphocytes producing the respective cytokines. The laboratory ranges of production of intracellular cytokines were based on *ad hoc* statistical analysis of the control group, with only 5% normal controls exceeding the range. These ranges were 18–68% for IL-2-, 22–77% for TNF-α-, 15–50% for IFN-γ- and 1–6% for IL-4-producing cells.

Phagocytosis

Whole blood was mixed with anticoagulant lithium heparin in tubes. The suspension of heat-killed (90°C/1 h) *E. coli* (strain K12) was obtained from EXBIO, Prague, Czech Republic, diluted in 0.9% NaCl to the concentration of 1 x 10^9/ml bacteria. A solution of dihydrorhodamine 123 (DHR123, Molecular Probes, Carlsbad, CA) was prepared by dissolving 10 mg of the substance in 26.2 ml dimethylformamide (DMF, Sigma-Aldrich, St. Louis, MO). The solution was aliquoted (per 50 μl)

Humlova.qxp 4.8.2006 10:03 Stránka 50

and stored at –20°C. The 0.11 mM solution used was prepared by adding 500 μl of 0.9% NaCl. Test samples were prepared by mixing 25 μl of whole blood with 10 μl *E. coli* suspension and 5 μl DHR123 in polypropylene tubes. Controls were prepared for each sample by mixing 25 μl of whole blood with 10 μl of 0.9% NaCl and 5 μl DHR123 in polypropylene tubes. Test samples and controls were incubated at 37°C for 45 min. Thereafter, 1 ml of 1x FACS™ Lysing Solution (Becton Dickinson Biosciences) was added to lyse erythrocytes. The remaining cells were washed twice with PBS. Polymorphonuclear cells ingest dead bacteria, which is followed by production of oxygen radicals. Free radicals convert DHR123 to rhodamine 123. The intensity of fluorescence of rhodamine 123 was determined by flow cytometry using FACS Calibur Becton Dickinson at 525 nm. The stimulatory index (SI) was the ratio between a control and a test sample. The standard laboratory referential ranges are 80–90% for *E. coli* ingestion and 20–80% for the SI.

Virus antibody assays

Antibodies were determined against the following herpesviruses: the herpes simplex virus type 1 and type 2 (HSV 1 and 2, IgG and IgM), the varicella-zoster virus (VZV, IgG and IgM), the human cytomegalovirus (CMV, IgG and IgM) and the Epstein-Barr virus (EBV, IgG and IgM against virus capsid antigen, VCA, IgG against virus nuclear antigen 1, EBNA1, and early antigens, EA- R+D); IgG and IgM against CMV, IgG against HSV, VZV and EBV (anti-VCA and anti-EBNA1) were detected in ELISA using commercial diagnostic kits (ETI-CYTOK-G and ETI-CYTOK-M reverse, DiaSorin, Saluggia, Italy, Herpes IgGII-EIA and Varicella zoster IgGII-EIA, Denka Seiken, Tokyo, Japan, EIA Viditest anti-VCA IgG and EIA Viditest anti-EBNA1 IgG, Vidia, Vestec, Czech Republic, respectively). In all instances the manufacturers´ instructions were followed. IgM antibodies against HSV 1+2 and VZV were determined by the indirect immunofluorescence tests in human diploid fibroblasts (LEP) infected with the respective viruses and fixed with acetone as described previously (Plummer, 1973). IgM antibodies against EBV were detected by indirect immunofluorescence using P3HR-1 cells as described previously (Roubalová et al., 1988). For detection of IgG antibodies against the early antigens of EBV (EA R+D), induced Raji cells were used as described previously (Anisimová et al., 1984). In all tests sera were diluted 1 : 10. In addition, antibodies to six human papillomaviruses (HPV), namely types 6, 11, 16, 18, 31 and 33 were determined using ELISA and virus-like

Table 1. Characteristics of patients enrolled

No.	Sex	Age	WBC x 109/l	Hgb g/dl	Trc x 109/l	Cytogenetic investigation	Molecular configuration
1	M	52	383.00	7.10	444.00	Ph+	b2a2
2	F	24	36.51	13.30	1613.00	Ph+	b3a2
3	M	60	239.00	8.30	2245.00	Ph+	b3a2
4	F	49	302.60	8.90	413.00	Ph+	b3a2
5	F	57	120.00	13.20	226.00	Ph+	b3a2
6	M	35	21.89	16.80	327.00	Ph+	b2a2
7	M	55	198.50	11.00	550.00	Ph+	b2a2
8	F	49	30.09	12.90	539.00	Ph+	b3a2
9	M	28	99.71	14.20	169.00	Ph+	b3a2
10	F	55	77.79	12.40	357.00	Ph+	b2a2
11	F	30	107.50	11.70	276.00	Ph+	b2a2
12	M	47	16.35	13.60	595.00	Ph+	b2a2
13	F	43	101.40	10.10	421.00	Ph+	b3a2
14	F	72	58.35	12.20	404.00	Ph+	b3a2
15	F	39	26.86	14.40	464.00	Ph+	b3a2
16	M	44	439.20	8.10	184.00	Ph+	b3a2
17	M	48	136.30	10.40	981.00	Ph+	b3a2
18	F	25	340.00	6.80	434.00	Ph+	b3a2
19	F	39	91.26	7.50	114.00	Ph+	b2a2
20	F	34	28.52	13.50	203.00	Ph+	b3a2
21	F	34	328.6	8.80	614.00	Ph+	b3a2
22	F	57	16.79	10.90	1895.00	Ph+	b3a2
23	M	30	266.3	9.80	768.00	Ph+	b3a2
24	F	57	82.55	10.70	677.00	Ph+	b3a2

WBC, white blood cell count; Hbg, hemoglobin; and Trc, thrombocyte count.

Humlova.qxp 4.8.2006 10:03 Stránka 51

particles (VLPs). The VLPs were prepared and the tests were performed as described previously (Hamšíková et al., 1998).

Statistical methods

To compare the differences in means of findings in the patients and the control subjects, the T-tests with Welch correction for heterogeneity, if present, were used (GraphPad InStat, version 3.00). The differences were called "significant" if the observed significance level was less than 5% and "highly significant" if it was less than 1%. To quantify the relationship between some of the observed tests, the pair-wise Pearson correlations were compared together with tests for their differences from zero. The differences at the 5% or 1% significance levels were assigned in the respective table by asterisk* or double asterisk**, respectively.

Results

The clinical characteristics of the patients and the basic laboratory findings as detected at their enrolment in the study, i.e. prior to the start of any anti-leukaemic therapy, are listed in Table 1. Fifteen were females and nine were males. All patients were Ph^+. Molecular tests detected b3a2 fusion in 17 patients and b2a2 fusion in 7 patients. No extramedullary involvement was found in any patient. In 23 patients who were considered for stem cell transplantation, HLA typing was performed. The patients' HLA profiles corresponded well with the distribution of HLA types in the population of the Czech Republic (results not shown). For a further, more detailed analysis the subjects were divided into two subgroups according to age, group I (24–44 years) and group II (45–72 years).

Immunoglobulins

The immunoglobulin levels determined are summarized in Table 2. As may be seen, a decrease of total IgG was found in two (8.5%) patients and an increase also in two patients. In the control group, IgG levels were also raised in two subjects. The mean levels of total IgG were nearly equal in the patients' group and the control group. In the IgG subclasses no differences between both groups were found, either. In three (12.5%) patients but in no control subject we observed decreased levels of IgA. The difference in IgA levels between the patients and the control subjects was statistically significant. The levels of IgM were increased in three (12.5%) control subjects but only in one patient. Comparison of Ig levels in age groups I and II and men and women did not indicate any marked differences (results not shown).

Presence of autoantibodies

Antibodies against thyroid peroxidase and thyreoglobulin were detected somewhat more frequently in normal controls than in patients, as shown in Table 3. In the patients' group we detected autoantibodies more frequently in females (nine cases, i.e 60%) than in males (three cases, 33%) (results not shown). Antibodies against smooth muscles (SMAb) were detected in two patients and two control subjects. We did not detect any antibodies against endomysium nor any antimitochondrial antibodies in either the patients or the controls.

Complement C3 and C4 components

Findings relating to the C3 and C4 complement components are shown in Table 4. We observed a decrease in the C3 level in two (8.3%) patients and one control subject and increased C4 levels in five (20.8%) other patients but in no control subject. The difference in the C4 levels between patients and controls was statistically significant.

Table 2. Immunoglobulin levels in patients and controls

Group	**IgG (total)**	**P**	**I/D**[b]	**IgG$_1$**	**P**	**I/D**	**IgG$_2$**	**P**	**I/D**	**IgG$_3$**	**P**	**I/D**	**IgG$_4$**	**P**	**I/D**
Patients	10.52 ±2.63[a]		(2/2)	6.88±1.83		(0/3)	3.38±1.20		(0/1)	0.36±0.23		(0/4)	0.51±0.48		(0/3)
		0.632			0.986			0.550			0.807			0.982	
Controls	10.86±2.06		(2/0)	6.89±1.25		(0/1)	3.61±1.43		(0/1)	0.37±0.15		(0/2)	0.51±0.42		(0/2)
Range	(6.90-14.00)			(4.90-11.4)			(1.50-6.40)			(0.20-1.10)			(0.08-1.40)		

Group	**IgA**	**P**	**I/D**	**IgM**	**P**	**I/D**
Patients	1.34±0.51		(0/3)	1.16±0.68		(1/0)
		0.018			0.348	
Controls	1.84±0.82		(1/0)	1.35±0.69		(3/0)
Range	(0.70-3.70)			(0.34-2.40)		

[a] The levels are expressed in g/l.

[b] In parentheses numbers of patients and controls with increased or decreased (**I/D)** Ig levels in comparison with the standard laboratory range (see Material and Methods)

Humlova.qxp 4.8.2006 10:03 Stránka 52

Table 3. Autoantibodies in patients and controls

Group	Anti TPO	Anti TG	ANAb	ANCAb	AMAb	endo A	endo G	SMAb
Patients	4 (16.7)[a]	2 (8.3)	3 (12.5)	1 (4.2)	0	0	0	2 (8.3)
Controls	8 (33.3)	5 (20.8)	3 (12.5)	0	0	0	0	2 (8.3)

[a] Numbers and percentage (in parentheses) of patients and controls positive for the respective antibodies
For explanation of the abbreviations see Material and Methods.

Table 4. Levels of complement components, CRP and IL-6 in patients and controls

Group	C3[a]	P	I/D[b]	C4	P	I/D	CRP	P	I/D	IL-6	P	I/D
Patients	1.01±0.19		(0/2)	0.31±0.21		(5/0)	20.33±28.05		(15/0)	5.97±3.24		(0/2)
		0.277			**0.038**			**0.011**			**0.0003**	
Controls	0.95±0.16		(0/1)	0.21±0.07		(0/0)	4.15±2.48		(3/0)	3.04±0.51		(0/0)
Range	(0.75-1.40)			(0.10-0.34)			(0.00-5.00)			(3.13-12.5)		

[a] The levels of C3 and C4 are expressed in g/l, CRP in mg/l and IL-6 in mg/l.

[b] In parentheses numbers of patients and controls with increased or decreased (I/D) levels in comparison with standard laboratory levels (see Material and Methods)

Table 5. Subpopulations of lymphocytes in patients and controls

Group	$CD3^+$	$CD4^+$	$CD8^+$	$CD19^+$	$CD56,CD16^+$
Patients	61.23±32.26[a]	48.25±19.4	21.40±10.7	12.9±7.7	16.9±.8.3
Controls	73.37±7.68	48.82±7.17	23.20±5.41	12.2±3.47	15.8±6.75
Range	(59-84)	(25-59)	(19-48)	(6-22)	(6-30)

[a] The figures indicate the percentages of cells positive for the respective surface CDs. The sum of lymphocytes reactive with either of CD4, CD8, CD19, CD16 and CD56 antibodies was considered 100%.

CRP and IL-6

The results are also shown in Table 4. The most striking difference between the two groups of subjects was found in the occurrence of increased levels of the CRP. We detected increased levels of CRP in 15 (62.5%) patients (with only one of them exhibiting clinical signs of inflammation), but only in three (12.5%) controls. This difference was statistically highly significant. The levels were more frequently increased in the elder patients (10 subjects, i.e. 83.3%) than in the younger ones (six subjects, i.e. 50%), but there was no difference between males and females (results not shown). The IL-6 levels were likewise higher in the patients than in the controls, although they only rarely exceeded the standard laboratory range. The difference in the IL-6 levels between patients and controls was statistically highly significant.

Lymphocyte subpopulations

The analysis of lymphocyte subpopulations was difficult because of the presence of high numbers of leukaemic cells in the patients. The percentage distribution of cells stained with antibodies against the different lymphocyte surface antigens are shown in Table 5. It may be seen that the distribution of the different lymphocyte subclasses in the patients and the controls was similar. Still, these data should be interpreted with caution because the conditions for analysis of the materials from the two groups were not equal: in the patient group there was an excess of leukaemic cells making precise measurement of lymphocyte subpopulations more difficult than in the control group. There were no significant differences between males and females nor between the younger and elder subjects (results not shown).

Intracellular production of cytokines

Intracellular cytokine production by stimulated $CD3^+$ lymphocytes was tested in 19 patients and their matched controls (Table 6). A decrease in IL-2 production was found in nine (47.4%) patients, TNF-α in seven (36.8%), IFN-γ in five (26.3%) patients and IL-4 in one patient (5.3%). The corresponding figures for the decrease in the control group were 1, 0, 0 and 0, respectively. In terms of the mean percentages of positive cells, the differences between the patients and the controls were highly significant in the case of IL-2- and TNF-α- but not in the case of IFN-γ-positive cells. The percentages of IL-4-positive cells were comparable in both groups.

Phagocytosis

The results of testing *E. coli* ingestion by polymorphonuclear cells of the patients and the controls are

Humlova.qxp 4.8.2006 10:03 Stránka 53

Table 6. Intracellular cytokine production by stimulated CD3$^+$ cells

Group	**IL-2**	**P**	**I/D**[b]	**TNF-α**	**P**	**I/D**	**IFN-γ**	**P**	**I/D**	**IL-4**	**P**	**I/D**
Patients	21.33±15.91[a]		(0/9)	31.82±17.54		(0/7)	24.99±15.21		(1/5)	2.77±1.53		(0/1)
		<0.0001			**0.0009**			0.18			0.25	
Controls	42.59±13.49		(0/1)	49.37±13.86		(1/0)	30.53±8.68		(1/0)	3.33±1.55		(2/0)
Range	(18-68)			(22-77)			(15-50)			(1-6)		

[a] The figures indicate the percentages of stimulated CD3+ cells producing intracellular cytokines. The total of 19 patients and 19 matched controls were tested.

[b] In parentheses numbers of patients and controls with increased or decreased (I/D) number of cells in comparison with the standard laboratory range (see Material and Methods)

Table 7. Phagocytosis by polymorphonuclears from patients and controls

Group	***E. coli* IN**	**P**	**I/D**[b]	***E. coli* SI**	**P**	**I/D**
Patients	97.96±1.59[a]		(13/0)	23.46±16.75		(0/11)
		0.08			**<0.0001**	
Controls	96.80±2.61		(7/0)	56.66±21.92		(4/2)
Range	(80-90)			(20-80)		

[a] The figures indicate the percentage of polymorphonuclears ingesting E. coli (IN) and reacting after their ingestion (SI)

[b] In parentheses numbers of patients and controls with increased or decreased (I/D) number of cells in comparison with the standard laboratory range (see Material and Methods)

Table 8. Antibodies against human herpesviruses and papillomaviruses in patients and controls

Group	HSV 1+2		VZV		CMV		EBV				HPV type					
							VCA		EBNA1	EA	6	11	16	18	31	33
	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgG						
Patients	91.7 [a]	0	100	0	75	8.3	100	0	100	20.8	41.7	8.3	12.5	12.5	8.5	8.5
Controls	71.8	0	95.8	0	62.5	0	100	0	91.5	20.8	25	0	0	8-V	12.5	8.5

[a] The figures indicate the percentages of sera reactive with the respective antigens. Mean titres were as follows (patients/controls): HSV 1+2 – 528/400, VZV – 309/400, CMV – 450/360, EBV VCA - 167/160, EBV EBNA1 - 241/260. In the other tests the results were classified as positive or negative.

shown in Table 7. The phagocytic activity was comparable in both groups. However, the *E. coli* stimulatory profile was more frequently decreased in the patients than in the controls. This difference was statistically highly significant.

Antibodies against herpesviruses and papillomaviruses

To ascertain whether onset of the disease was associated with immunosuppression, which might result in activation of latent and/or persistent virus infections, we tested sera from the patients and control subjects for antibodies to five human herpesviruses and six human papillomaviruses. The results are shown in Table 8. No significant differences were found between patients and controls, either in the distribution of antibodies or in their titres. However, it may be noteworthy that CMV IgM antibodies were detected in two patients but in none of the controls.

Correlations between the various laboratory findings

Correlations between the different individual findings in the patients are shown in Table 9. Only data in which a statistically significant association was revealed are presented. Though many of the correlations could be expected, some of the findings may be of interest. Thus, e.g., the WBC count and the severity of anaemia were strongly associated with the IL-6 level and with a decrease of the *E. coli* SI. Total IgG was associated with the C3 level and *E. coli* ingestion. The latter correlation was also found for IgG_1 but not for the other IgG subclasses, suggesting that only IgG_1 and no other component of the IgG family was involved in the association. On the other hand, the level of IgG_4 but of no other IgG subclass levels was strongly positively correlated with the IL-6 level and weakly, but significantly, negatively with the *E. coli* SI. Significant correlations were

Humlova.qxp 4.8.2006 10:03 Stránka 54

Table 9. Correlation between different laboratory findings

	WBC	HGB	TRC	IgG (total)	IgG_1	IgG_2	IgG_3	IgG_4	IgA	IgM	C3	C4	CRP	IL-6	CD3+				*E.coli* IN	*E.coli* SI
															IL-2	TNF-α	IFN-γ	IL-4		
WBC	1.0	*.-776***												.852**						*-.638***
HGB		1.0						*-.421**	.445*					*-.719***						.634**
TRC			1.0	.467*	.410*	.495*														
IgG (total)				1.0	.926**	.824**					.409*								.557**	
IgG_1					1.0	.626**													.532**	
IgG_2						1.0		.443*							.511*					
IgG_3							1.0			.400*										
IgG_4								1.0						.555**						*-.480**
IgA									1.0		.545**					.494*	.459*	.482*		
IgM										1.0										
C3											1.0								.667**	
C4												1.0								
CRP													1.0			*-.481*				
IL-6														1.0						*-.461**
CD3 IL-2															1.0	.534*				
CD3 TNF-α																1.0	.781**			
CD3 IFN-γ																	1.0			
CD3 IL-4																		1.0		
E.coli IN																			1.0	
E.coli SI																				1.0

The figures indicate correlation indices, * $P < 0.05$, ** $P < 0.01$. Negative correlations are indicated in italics and marked with symbol -. Explanation of abbreviations: WBC – white blood cell count, HBL – haemoglobin concentration, TRC – thrombocyte count, C3 – C3 component of complement, C4 – C4 component of complement, CD3 IL-2 – $CD3^+$ cells producing intracellular interleukin 2, CD3 TNF-α – $CD3^+$ cells producing intracellular tumour necrosis factor a, CD3 IFN-γ – $CD3^+$ cells producing intracellular interferon g, CD3 IL-4 – $CD3^+$ cells producing intracellular interleukin 4, *E.coli* IN – polymorphonuclears ingesting killed *E. coli, E. coli* SI – polymorphonuclears stimulated after ingestion of killed *E. coli.*

Humlova.qxp 4.8.2006 10:03 Stránka 55

found between IgA and C3 levels and between IgA levels and the counts of $CD3^+$ cells that produced either TNF-α, IFN-γ or IL-4. CRP levels correlated negatively only with the counts of $CD3^+$ cells producing TNF-α. Surprisingly, the raised levels of CRP did not correlate with the IL-6 levels. IL-6, in addition to the correlations already mentioned, negatively correlated with *E. coli* SI, indicating that the polymorphonuclears of the patients with increased IL-6 level had a lower tendency to react after ingestion of the bacteria. Among the $CD3^+$ cells, significant positive correlations were found between the IL-2- and TNF-α-producing cells, and between the latter cells and IFN-γ-producing cells.

Discussion

In the present undertaking we tested CML patients for a number of immunological parameters, to the best of our knowledge more than in any prior study. We expect the follow-up to provide some new information that might be utilized in monitoring the course of the disease and the efficacy of its therapy and in developing a rational strategy for the future immunotherapy of this deadly disease. To validate any possible deviations from the norm and the methods used we also included in the study a group of normal subjects matched with the patients by sex and age.

We did not observe any marked differences in IgG and IgM levels between the patients and the controls, which is in line with a previous observation (Solanki et al., 1990). However, decreased levels of IgA were observed in three patients but in none of the control subjects and, in general, the levels of IgA were lower in the patients than in the controls. They significantly correlated with lower counts of $CD3^+$ cells producing, after induction, several Th1 cytokines. It has been shown that decreased levels of IgA are frequently observed in patients with malignancies and, vice versa, that low levels of IgA predispose to malignant diseases (Shacklefford and McAlister, 1975; Mellemkjaer et al., 2002). Significantly altered complement levels have been found at the blastic stage of the disease, whereas at remission, normal values have been recorded (Minh et al., 1983). In our study, we found an increased level of C4 in more than one fifth of the patients. This indicates that the alterations of at least this component of complement can be seen in the early stage of the disease. It may be of interest that the increased C4 levels did not correlate with any other immunological parameter tested.

The most striking difference between patients and controls was in the occurrence of increased levels of CRP. We detected them in over 60% of patients. In only one patient there were clinical signs of inflammation at the time of sampling; however, the presence of microinflammation processes cannot be ruled out in other patients. It should be recalled that another acute phase reactant, orosomucoid, has been reported to be elevated in CML patients and its level reportedly correlated with the white blood cell (WBC) count, the stage of the disease and also the CRP levels (Le Coutre et al., 2002). However, in our patients we did not observe any correlation between increased CRP levels and the WBC count or any other findings. The only statistically significant association found was a negative correlation between CRP levels and stimulated $CD3^+$ cells producing TNF-α. It will be an aim of the follow-up to evaluate any relationship between the raised CRP levels and the further course of the disease and its response to therapy. It is not easy to interpret why the CRP levels were increased, especially in view of the recent results on the properties of this substance (Du Clos and Mold, 2004; Szalai, 2004). Apparently, CRP plays roles in both innate and adaptive immunity. At the high acute state levels that are associated with IL-10 induction, it suppresses inflammation, and there are indications that it protects from certain autoimmune diseases, possibly through induction of suppressive regulatory cells. At the moderately increased levels, seen in the high proportion of the CML patients tested in the present study, it may have an opposite effect by activating complement and inducing proinflammatory cytokines (Du Clos and Mold, 2004). It is suspected that this disparity between the biological effects of high and low levels of CRP may be associated with the involvement of different receptors with varying affinity for the substance. Possibly, the follow-up of the patients and the ever increasing knowledge on the biology of CRP will help elucidate the relationship between its levels and the course of CML. Since it is known that IL-6 is a CRP inducer (Mahmoud and Rivera, 2002), it was rather surprising to find that in individual patients the CRP and IL-6 levels did not correlate, indicating that the increase of these substances in CML patients is at least partially IL-6 independent. The finding that CRP induction by IL-1 and IL-6 are controlled differently (Kleeman et al., 2003) may provide explanation for the present observation. As concerns the IL-6 increase, observed already in the past for CML (Anand et al., 1998) but not for some other leukaemias (Inoue et al., 1994), the significance of this phenomenon is unclear. With respect to the present discussion it may be of interest that IL-6 was able to inhibit p53-induced apoptosis of a myeloid cell line (Yonish-Rouach et al., 1991) and suppress phosphorylation of the retinoblastoma protein in haematopoietic cells (Resnitzky et al., 1992). Increased levels of IL-6 have also been detected in myeloma patients (Hirano, 1992) and in adult T-cell leukaemia/lymphoma patients, in whom they correlated with adverse clinical features and a bad prognosis (Yamamura et al., 1998). Similarly, it has been suggested that serum IL-6 levels may serve as a prognostic marker in CML (Anand et al., 1998).

Humlova.qxp 4.8.2006 10:03 Stránka 56

It is well known that in CML patients treated with IFN-α or hydroxyurea, an autoimmune reactivity develops (Tothova et al., 2002; Dacey and Callen, 2003; Steegmann et al., 2003a). We tried to map the autoimmune terrain before any therapy was started. Positive reactions were observed more frequently in the control group than in the patients, more so in females than in males, and more so in the elder women than in the younger ones. These findings further support the present concept that the autoimmune reactivity so frequently seen in CML patients is not induced by the disease itself (at least in its early stage), but by the therapies administered.

As in other malignancies, it is very important to understand the state of T-cell immune reactivity in CML patients. It seemed of special importance to assess the T-cell repertoire in untreated patients. Our efforts were limited to analysing the T-lymphocyte subpopulations and the production of intracellular cytokines. For technical reasons the results obtained in these tests must be evaluated with caution. However, they suggest that the ratios between the different subclasses of lymphocytes were not significantly altered in our CML patients. Further tests revealed a decrease of intracellular cytokine production in stimulated $CD3^+$ cells. We detected decreased production of at least one of the cytokines tested in the majority of patients. The most frequent deviation from the norm was a reduction of IL-2-producing cells**.** Our data are in line with the earlier observation by Reuben et al. (2000), who reported a decrease of production of IL-2-, TNF-α- and IFN-γ-producing cells in CML patients in the chronic phase of the disease. However, they observed a restoration of their synthesis when remission had been achieved. Also Kiani et al. (2003) were unable to detect any differences in the intrinsic cytokine-producing capacity of T-helper cells in a group of CML patients in haematological remission and matched control subjects. Thus, it seems that this deficiency is limited to the untreated and unsuccessfully treated patients.

We also tested the phagocytic function of polymorphonuclear cells in CML patients. When comparing patients with controls, we observed nearly the same ingestion activity in both groups, but the *E. coli* SI measured as oxidative burst was significantly lower in the patient group. The suppression of the SI strongly correlated with WBC counts and the severity of anaemia. These findings seem to reflect a reduced functional activity of the patients' polymorphonuclears.

In other tests the presence of antibodies against human herpesviruses and papillomaviruses was determined. These viruses induce latent and/or persistent infections and may be activated under immunosuppression. The results did not reveal any significant difference in the prevalence of antibodies and their titres between the patients and controls, suggesting that at least in the initial stage of the disease the altered immune reactivity is not reflected by activation of herpetic or papillomavirus infections.

To summarize, a number of deviations from the norm were detected in the immune reactivity of CML patients before the start of therapy. The pattern of these deviations was different in individual patients. The most frequent alterations observed were an increased level of CRP, reduced numbers of cells with intrinsic production of cytokines, more so in the case of IL-2 and TNF-α than IFN-γ and IL-4, and a decreased *E. coli* SI. While some of the correlations between the different findings could be expected and may be explained easily, some other were rather surprising and are not readily understood at present. It will be the purpose of the follow-up under way to determine whether and how the alterations detected will affect the subsequent course of the disease and how the individual markers of immunity and their co-variations will change in response to the therapy and to the changing clinical condition.

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Humlova.qxp 4.8.2006 10:03 Stránka 57

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