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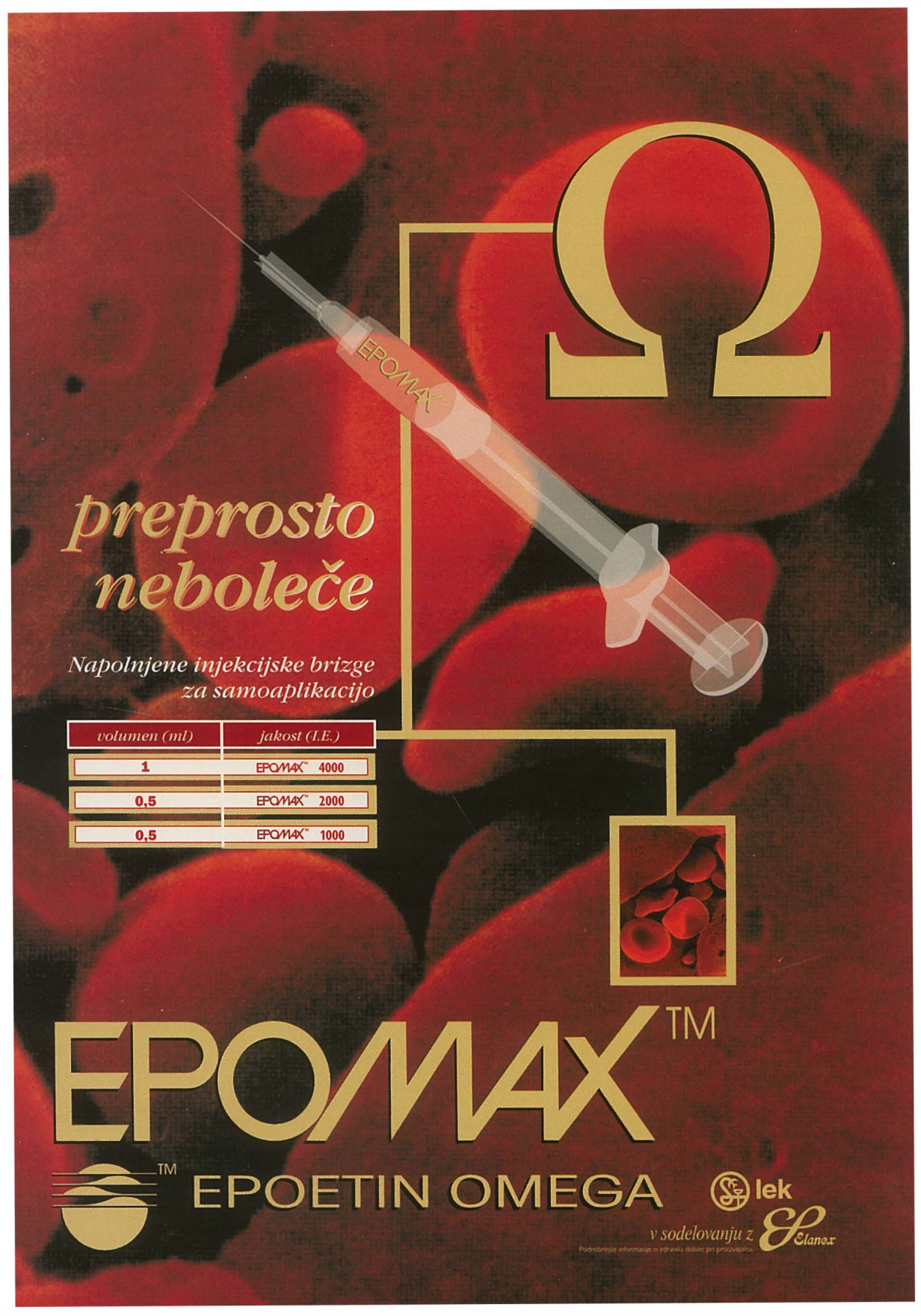
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Obstruction of the duodenum due to a billiary calculus (Bouveret's syndrome)

Aleksandar Včev[1], Jerko Barbić[1], Andrijana Včev[1], Damir Kovačić[2], Miroslav Vegar[2]

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Duodenal obstruction by a gallstone is a very uncommon clinical condition. The gallstone obstruction of duodenum is usually discovered by gastrointestinal endoscopy or x-ray examination of the upper gastrointestinal tract. Here, we report the case of a 76-year-old woman with a gastric outlet obstruction due to gallstone. The obstruction was initially diagnosed by the ultrasound of the upper abdomen and was later confirmed by gastroscopy. The attempt of endoscopic extraction of gallstone from the duodenum was unsuccessful and was consequently removed by surgical procedure.

Key words: choletiasis-complications; duodenal obstruction

Introduction

The intestinal obstruction secondary to a gallstone is an unusual complication of cholelithiasis and accounts for 1% of all cases of intestinal obstruction, however the incidence of this condition rises up to 25% in those over age 70.[1] The stone usually lodges at the terminal ileum, the narrowest portion of normal gut, but stones can be impacted in pylorus, duodenum, jejunum or colon. The duodenal obstruction by a gallstone is a very rare condition.[2,3] In recent years, diagnosis has been facilitated by endoscopy and several cases have been reported.[4-8] Here, we report a case of duodenal obstruction in which the diagnosis of gallstone obstruction was made endoscopically and relieved by surgical procedure.

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Case report

A 76 year-old woman was admitted to hospital because of nausea and vomiting. The nausea and vomiting were lasting for the last three weeks. Since than, she lost appetite and 4 kg of body weight. During last seven days vomiting was persistent. The patient stopped eating and she just drank milk and yoghurt.

The patient had been well until about six months earlier when she had pain in the upper abdomen. At that time, the pain was treated symptomatically with H_2-receptor antagonist, antacids and diet. The peptic ulcer disease as the cause of this pain was

not confirmed by endoscopic examination at that time. However, with this treatment she felt better until the admission to our hospital. She had no previous history of cholelithiasis attacks.

At the admission, physical examination disclosed dehydration, tenderness and epigastric distension. Laboratory tests were performed. The laboratory analysis revealed normal liver function and prerenal azotemia with hypokalemia, hypochloremia and alkalosis. Ultrasound examination of the upper abdomen was performed. It showed a high density mass of 5 cm in diameter at the projection of bulbus duodeni. The endoscopic examination revealed one large, round, hard, black-green mass projecting through pylorus (Figure 1). Moreover, by endoscopic examination we observed an ulcer of the anterior wall of the duodenal bulb with bulb deformation, dilatation of stomach and reflux esophagitis "Savari Miller gr III". This mass was interpreted as a gallstone obstructing duodenum and the x-ray examination of the upper abdomen was considered unnecessary.

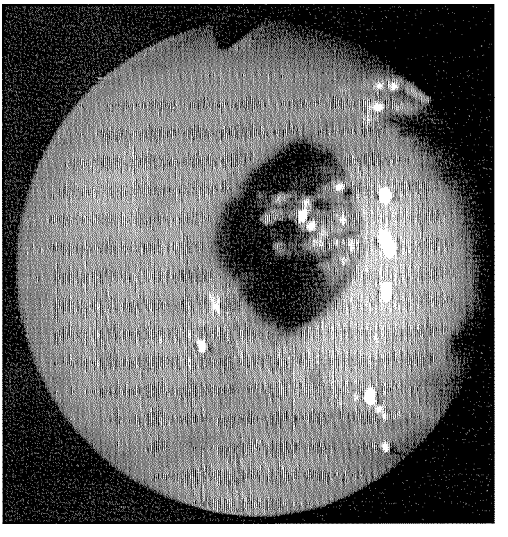

Figure 1. Gallstone projecting through pylorus at the endoscopy.

We were not able to remove the gallstone by endoscopic mechanical lithotripsy. After correction of dehydration, metabolic alkalosis and hypokalemia, a successful removal was performed by surgical procedure. The surgical procedure was performed by enterotomy proximal to the stone, and removing offending calculi with closure of the intestine. The patient's condition allowed us to perform concomitant cholecystectomy with fistula closure. The opening of cholecystoenteric fistula was on the anterior wall of the duodenal bulb. The patient's post-operative condition was good.

Discussion

Bouveret described duodenal obstruction due to biliary stone in 1896. Bouveret's syndrome is characterised by signs of ileus like abdominal pain, vomiting and dehydration. Patients usually had a prior history of symptomatic biliary tract disease. However, the patient has not mentioned abdominal pain, biliary colic as well as fever. Fifty-eight cases of this rare clinical entity were described by Simonian in 1968[2] and since then others[4-8] have reported several new cases. It is important to note that most reported new cases were diagnosed by endoscopic examination of stomach and duodenum. Bouveret's syndrome may occur in 1% to 3% of patients with cholecystoenteric fistula.[9] The occurrence of cholecystoenteric fistulas has been reported in 0.09% to 3.2% of patients with biliary disease.[9] Gallstone ileus is a complication in 0.3% to 0.5% of all cases of cholelithisais.[3] It is important to have in mind that ileus could be caused by a gallstone. Moreover, the incidence of gallstone ileus rises significantly in patients over 65 years of age.[1,3] Here, we showed that the ultrasound examination of the upper abdomen could give the first indication of a gallstone. This is to our knowledge the first report that this easy and fast diagnostic

approach can be used. The usual initial diagnostic step is the x-ray examination of the abdomen, which must comprise, in differential diagnosis, other causes of intestinal obstruction like duodenal neoplasm, duodenal polyp and finally foreign body.[2] However, here the final diagnosis of Bouveret's syndrome was established by the endoscopic examination of the stomach, as it was described by others.[4-8] It is possible that the stone may be endoscopically removed or, at least, manipulated in stomach so that after relieving the obstruction, the patients will go for definitive surgical treatment with fistula closing and cholecystectomy, especially in patients in good health condition where is likely that gallstone ileus occur again.[5,6] Surgical procedure can be considered as only definitive treatment for Bouveret's syndrome.

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Radiol Oncol 1998; 32(2): 165-9.

A rare case of symmetric bifemoral fractures in battered child syndrome and overview over the literature

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We report on a 1-year-old girl who was suspected for battered child syndrome. A conventional X-ray showed a symmetric bifemoral fracture close to the distal growth plates and a l eftsided parietal fracture. However, bone scintigraphy revealed only a slight symmetric widening of the corresponding growth zones in the axial direction when compared with the proximal tibial growth areas while the parietal fracture was missed. A diagnostic difficulty is stressed, and a criteria for bone scanning in battered child syndrome are reviewed.

Key words: battered child syndrome, femoral fractures; radionuclide imaging

Case history and clinical findings

A one-year-old girl with a history of fever and diarrhea for three days was admitted to the children's hospital. The girl's left leg was in a cast due to a distal femoral fracture. She suffered from pain in her right leg so that she refused to get in a standing position for a week. This drew attention to a suspected fracture of the right femur. Moreover, she had several hematomas of different age all over the body and a blood-crusted exanthema at the occiput. A bilateral periocular hematoma was noticed by her pediatrician three weeks ago which was explained by her mother to be caused by falling twice against a table. She was noticeably frightened about foreign people. Her general, nutritional and care conditions were poor. The examinations were undertaken in order to prove a suspected battered child syndrome.

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Laboratory results

C-reactive protein was markedly increased at 2.9 mg/dl (normal: < 0.8 mg/dl). All further standard laboratory findings, i.e. blood cell count of erythrocytes, leucocytes and platelets were in the normal range.

X-ray

An X-ray of both femurs showed a bilateral transverse fracture at both distal diaphyses in close relation to the corresponding growth plates: on the left side an older fracture with callus formation was evident and at the right side a new fracture without any callus formation was seen (Figure 1). A lateral X-ray of the

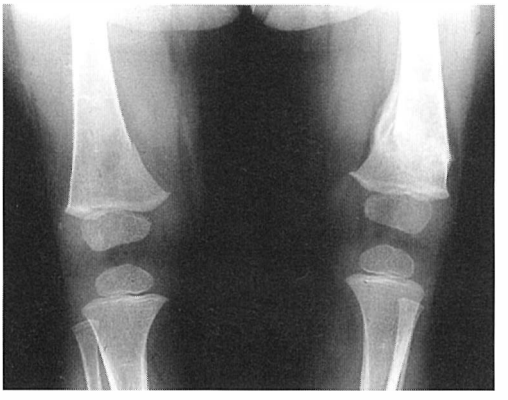

Figure 1. Anteroposterior X-ray of the knees showing an older transverse fracture with callus formation in the left distal femoral meta-diaphyseal region, and a fresh fracture without callus formation in the right distal femoral meta-diaphyseal region.

skull showed a large left-sided parietal fracture (Figure 2) without signs of a basocranial fracture. A computer tomography of the skull revealed no signs of cerebral or subdural hematoma. A chest X-ray showed a dislocation in the costo-vertebral junction of the 10^{th} left rib.

Bone scintigraphy

Conventional bone scintigraphy was performed 3 hours post i.v. injection of 200 MBq Tc-99m-HDP in anterior and posterior views using a double head gamma camera equipped with a low energy high resolution collimator. An increased tracer uptake was seen next to the distal femoral growth plates

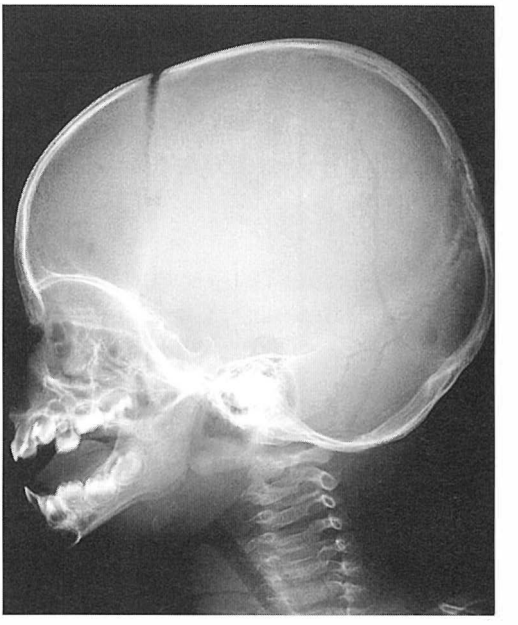

Figure 2. Lateral X-ray of the skull showing a left-sided parietal fracture.

on both sides corresponding to the fracture sites. Moreover, a slight widening of the distal femoral growth plates was observed when compared to the proximal tibial growth plates. Furthermore, a diffusely higher uptake in the diaphysis of the right femur was seen in this patient (Figure 3). Focal tracer uptake was evident at the costo-vertebral junction of the 10^{th} left rib. Marginally increased asymmetric tracer uptake was seen at the left side of the skull, but this was considered as normal since the patient's head was turned slightly to the left. No further spot images were performed.

Therapy

The right leg was stabilized by a cast. Since battered child syndrome was very likely by the results of all examinations the custody for the child was withdrawn from the parents.

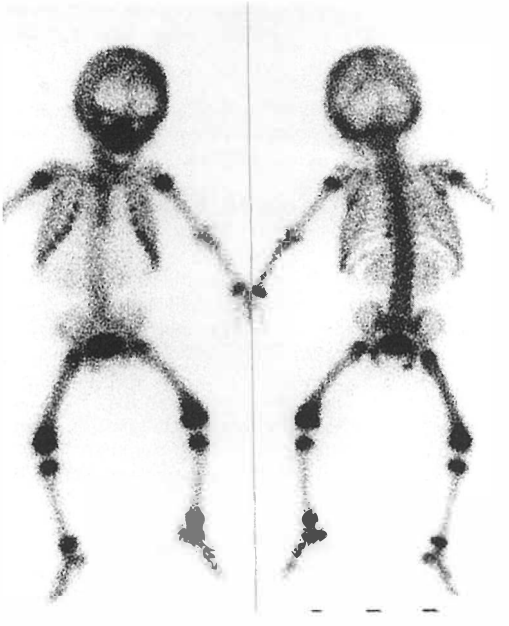

Figure 3. Conventional whole-body bone scintigraphy in anterior and posterior views showing increased tracer uptake of both distal growth plates of the left and right femur and bilateral slightly widened distal femoral growth plates as compared to proximal tibial growth plates. Furthermore, a diffusely higher uptake in the diaphysis of the right femur is seen in this patient. Moreover, focal tracer uptake is evident at the costo-vertebral junction of the left 10th rib. Marginal enlarged asymmetric tracer uptake in the left side of the skull.

Discussion

A child abuse and a battered child syndrome show an increasing incidence with a high estimated number of unreported cases in the last few years.[1,2] Characteristic clinical findings of a battered child syndrome are hematomas, retinal hemorrhage, skin burns and fractures of different ages situated predominantly in the skull, ribs and metaphyses of the long bones.[3-14] For forensic consequences a suspected battered child syndrome has to be proved carefully.[15-19] Thus, several diagnostic methods have been established as screening procedures in order to prove bone involvement, e.g. bone scintigraphy and X-ray studies.[20,21] However, there is still a high number of false- negative results both in scintigraphy and conventional radiologic examinations varying from 1–50%.[2,3,22-25] Therefore, a single diagnostic method is not sufficient to prove all osseus and abdominal lesions. Consequently, a variety of examinations, i.e. bone scintigraphy, X-ray, and computerised tomography may be necessary to sufficiently prove all lesions.[4,26,27,28]

The detection of metaphyseal fractures located near the growth plate by bone scintigraphy is difficult since there is an increased tracer uptake due to physiological activity in this region. Thus, an important diagnostic criterion in the detection of these fractures is an asymmetric tracer uptake. Nevertheless, a high number of false negative bone scans in fractures located close to the growth plates has been reported by several authors.[2,29]

In our patient bone scintigraphy yielded slightly enhanced tracer uptake of both distal femoral growth plates indicating increased bone activity in the fracture sites. Moreover, a slight widening of both distal femoral growth plates in axial direction was observed. Thus, the detection of symmetric bifemoral fractures in bone scan was hampered first by the cast on the girl's left leg which made an accurate symmetric positioning impossible, second by the localization of the fractures close to the growth plates which show physiologically increased tracer uptake, and third to a lack of side-related differences due to the bilateral occurence of the fractures. The only hint pointing out a symmetric bifemoral fracture was the widening of the distal femoral growth plates in axial direction when compared to the proximal tibial growth plates.[30] Furthermore, a diffusely higher uptake in the diaphysis of the right femur was seen in this patient which may be one of the typical presentations in a battered child.

As compared to conventional X-ray the parietal fracture of the skull showed only a slightly enhanced tracer uptake. This was considered normal since the patient's head

was turned slighly to the left. A high number of false-negative bone scans in the detection of skull fractures is well-known.[23,25] Focal tracer accumulation at the costovertebral junction of the 10th left rib was shown by a conventional X-ray to be related to a slight dislocation of the rib.

With respect to our findings we would like to emphasize some points that should be kept in mind in differential diagnosis of a battered child syndrome:

- Bone scintigraphy in these patients is both demanding and not frequently performed. Thus, a special attention should be paid to it.
- An exact symmetric positioning should be ensured in order to allow an accurate side-to-side comparison.
- Images with excellent count statistics should be acquired in order to enable the detection of the minor increased focal tracer uptake. Thus, sometimes the sedation of the patients may be necessary for the scintigraphic imaging to avoid misleading artefacts.
- In addition to a whole-body scintigraphy spot images should be obtained, e.g. lower extremities, vertebral spine, skull from lateral views or from a parietal top view.
- Even under most accurate conditions there is a decent number of false-negative results in either bone scintigraphy or conventional X-ray.
- Therefore, the final evaluation of bone lesions should include all lesions found either by the bone scintigraphy or by the conventional X-ray.

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review

Polymerase chain reaction procedures in the diagnosis of lymphoproliferative disorders

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Immunohistochemistry and molecular genetic techniques greatly contribute to our understanding of lymphoproliferative disease. The majority of lymphomatous lesions can be diagnosed by morphology alone but additional diagnostic tests have to be employed when cell lineage and clonality are not obvious. Morphologic distinction of Hodgkin's from non-Hodgkin's lymphoma, or of inflammatory lesions from malignant lymphoma, can be challenging. Non-random chromosomal translocations may help to recognize lymphoma subgroups with distinct biological characteristics. This review focuses on the polymerase chain reaction (PCR) techniques that have become an important diagnostic tool applicable to limited cellular material and paraffin-embedded tissues. The effectiveness of PCR in rearrangement analyses of T cell receptor g and immunoglobulin heavy chain genes is well documented. Some of the known translocations, such as t(14;18) or t(2;5) can be routinely assessed by genomic PCR or by reverse-transcription PCR. Those involving the bcl-1, bcl-6, and c-myc genes require more elaborate and sophisticated PCR procedures. Molecular genetic analyses by PCR, besides their immediate diagnostic value, bear the potential to identify new criteria for lymphoma diagnosis in conjunction with cytomorphology and immunophenotyping.

Key words: lymphoproliferative disorders-diagnosis; polymerase chain reaction; lymphoma-diagnosis

Introduction

Clonality of B cell proliferations, B or T lineage assignment, and maturation stage of lymphoid tumor cell populations are mainly established by immunophenotyping. This method largely fails to determine clonality in T cell tumors and to assign lineage to either very immature cells, which do not yet express these markers, or abnormally activated cells with loss of surface antigen expression. Lymphoma tissues may also be difficult to classify based on morphology and immunohistochemistry alone when the malignant clone is obscured by reactive lymphoid cells. The discovery of immunoglobulin (IG) gene

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rearrangements and the application of molecular probes for these genes has opened a new avenue to the diagnosis and biological understanding of lymphoproliferative disease. The discovery that rearrangement processes in T cell receptor (TCR) genes parallel the recombination events in immunoglobulin genes, led to the development of molecular tools applicable to the search for clonality in T-lymphoproliferative lesions.[1] Cloning of molecular breakpoints for tumor-specific chromosomal translocations permits the identification of a malignant clone using molecular probes for analysis of DNA simply extracted from lymphoid tumor tissue.[2] Data from molecular investigations with such probes now provide information about the origin of tumor lymphocytes as well as clues to molecular mechanisms involved in blastic transformation and tumor progression.

Gene rearrangement analyses by Southern blot procedures have become a valuable adjunct in the diagnosis of lymphoproliferative disorders.[3] This approach has serious limitations. The DNA has to be cut with several restriction enzymes. Hybridization with several probes is often required to obtain reliable and reproducible results. Large quantities (usually more than 20 µg) of high molecular weight DNA have to be extracted from fresh / fresh-frozen tissue or cell suspensions for these studies. The whole procedure takes several days, often weeks. Application of the polymerase chain reaction (PCR) techniques overcomes these limitations.[4] Specific DNA fragments can be rapidly amplified with oligonucleotide primers binding to both ends of a gene sequence of interest. Genomic DNA amplification techniques are not restricted to the analysis of fresh-frozen tissue samples with their limited morphological quality. DNA extracted from the more readily available formalin-fixed, paraffin-embedded tissue samples, even tiny areas of interest from a stained section or single cells with approximately 10 pg of DNA provide enough template for amplification.

This review focuses on well characterized PCR protocols for routine analysis of B and T cell neoplasms. Diagnostically important chromosomal aberrations will be considered and the advantages and limitations of molecular genetics in lymphoma diagnosis are discussed.

IG and TCR gene rearrangement

Mechanisms and methods

The only known rearranging genes are lymphocyte receptor genes for antigen, coding for either IG or TCR chains. Rearrangement involves random assembly of different variable (V), sometimes diversity (D), and joining (J) gene segments, which are discontinuously spread out within a chromosomal location in germ line configuration.[5] The recombinase activity is at least in part initiated by products of two recombinase activating genes, RAG1 and RAG2. Expression of these genes strictly correlates with V(D)J recombinase activity. Their transcripts occur in pre-B and pre-T cells, and are re-expressed during B cell selection in germinal centers where they may be involved in V gene replacement of rearranged IG genes.[6] A set of conserved nucleotides, heptamers and nonamers flanks the germline V, D, and J segments. They function as recombination signal sequences for V-D, D-J, or V-J joining, and are recognized by a recombinase enzyme system. These signal sequences are separated by a nonconserved spacer. The spacer situated 3' of the V or D gene segment is 21-23 bp long (about two turns of the DNA helix). It is 11-12 bp long (about one turn of the double helix) when located 5' of the D or J gene segment. Flanking sequences with a one-turn spacer signal can only rearrange to a two-turn signal. This probably ensures joining of

appropriate gene segments. Thus, one V and one J can recombine, but more than one D segment can join. TCR Vβ and Jβ heptamers, however, are virtually indistinguishable from those found next to immunoglobulin genes suggesting that the recognition devices for IG and TCR gene rearrangements are very similar. This explains the observation that T cells have occasionally D-J rearrangements of the IG heavy chain genes. Most commonly, the coding joint stays in the chromosome and a circular DNA molecule containing the signal joint and intervening sequences is excised. Intervening DNA stretches are retained if the two segments are joined in an opposite transcriptional orientation (conversion). At the coding ends, the joining is commonly imprecise. This happens through differential trimming of recombining gene termini by exonucleases and through duplication of one or two nucleotides at the recombination cleavage sites (P-nucleotides). Introduction of up to 15 nucleotides between V-D, D-D, D-J or V-J junctions in every possible random sequence generates non-template (N-) diversity. The enzyme terminal desoxynucleotidyl transferase (TdT) probably mediates addition of these nucleotides. N diversity contributes most significantly to the variability of the immune receptors, but it may also result in the generation of stop codons at the coding junctions.[7] In B cells, affinity maturation of the IG receptor to antigen in germinal centers is managed by extensive base substitutions in the rearranged V segments. Most of these mutations accumulate in the three complement-determining regions (CDRs). This process of somatic hypermutation apparently does not occur in TCR genes. Rearrangements involve different TCR and IG chains at different stages of lymphocyte ontogeny as defined by immunophenotyping. The first step in B cell development involves the D to J joining of the IG heavy chain genes (incomplete rearrangement) which precedes the V to DJ joining (complete rearrangement) forming a functional V region gene on one allele of the pre-B cell with cytoplasmic IGμ expression. Later during B cell ontogeny, the IG light chain (IGL) genes rearrange. Immature B cells, expressing either μk or μλ on the surface, result from the successful completion of heavy and light chain gene rearrangements. Similarly, a stepwise rearrangement of the immune receptor genes is found in cells committed to T lineage. Ninety to 95% of mature T cells carry the TCRαβ receptor on their surface, the remainder having TCRγδ chain heterodimers. Studies of precursor T-cell leukemia suggest that the TCRδ genes rearrange before the TCRγ genes. It is known that an incomplete DJ joining of the TCRβ chains is the next event and that a complete rearrangement of this gene locus follows. Finally, the TCRα chains rearrange. T cells expressing the TCRγδ receptors may not have rearranged their TCRα chain genes on both alleles, since the TCRd locus is nested between the Va and the Ja segments on chromosome 14q11, and rearrangement of these segments would result in a deletion of the TCRδ locus.

As a result of the maturation of lymphocyte progenitor cells, individual T cells with uniquely rearranged TCR genes and individual B cells with uniquely rearranged IG genes arise. Antigenic stimulation generates a polyclonal or oligoclonal lymphoproliferation under control of the immune system. Clonal populations will emerge if immune surveillance fails to control the lymphoproliferation. Clonality indicates autonomous growth of tumor cells which is an important diagnostic criterion for malignant lymphomas. TCR and IG gene rearrangement studies are therefore extremely valuable in the diagnosis of lymphoproliferative disease.

PCR primers for IG and TCR genes

PCR amplification requires less than 1 μg of template DNA. Degradation of the DNA as

present in formalin-fixed, paraffin-embedded material, stained sections or archival cytology smears does not seriously affect the reaction as long as the cell morphology is sufficiently preserved. Therefore, even small areas of interest in a stained tissue section can be scraped off and used as DNA source.[8] Rearranged IGH genes of single lymphoid cells from sections of fresh-frozen or fixed tissue specimens have been successfully amplified with V gene family-specific and consensus J region primers.[9] Prolonged over a week long with formalin or with B5 generally results in a less reliable amplification when compared to shorter formalin fixation times. Certain fixatives, such as Bouin's solution, or decalcification of tissue with ethylenediaminetetraacetic acid (EDTA) results in extensive DNA degradation so that, at best, only very short sequences can be amplified. Visualization of PCR amplification products on a high percentage agarose or a polyacrylamide gel normally identifies a clonal population, and rarely is Southern blot analysis or sequencing of the gene products necessary for routine applications. Because of its speed and simplicity, the PCR analysis is given priority for the diagnostic assistance in a difficult case. The rate of false-negative results is, however, higher than in Southern blot studies, so that the latter technique is still valuable in the case of unsatisfactory PCR results.

The CDR3 region of the VH gene segments, formed by the D gene segment and the V-D as well as the D-J junctions with the variable nucleotide deletions/insertions, can be amplified by PCR with primers that bind to consensus gene regions in IGH V framework regions (FR1, 2 or 3) and FR4 in the J regions.[10] Only complete VDJ rearrangements with the V and J sequences in the right orientation are amplified. Clonal incomplete DJ rearrangements, readily detectable by Southern blot using appropriate J and C region gene probes, cannot be amplified by PCR since the V regions are separated from the J segments by intervening sequences which are too long to be amplifiable. The use of other primers residing in the FR2 region is helpful in those cases where the FR3 primers fail to bind. V gene family-specific primers derived from the FR1 region are used in separate PCR reactions or combined in one so-called multiplex PCR, but they require a template DNA of reasonably good quality to allow for the longer PCR products (about 350 bp as compared to 120 bp when CDR3 region primers are used).

Similar to Southern Blot analyses, a background smear of amplified DNA is detected when polyclonal B cells are analyzed, since the length of the amplified fragment varies between individual B cells due to the different D gene sequences and the diversity of the flanking N regions. PCR products of one or two predominant sizes separated from the polyclonal background smear by gel electrophoresis are evidence of a clonal B cell population. This implies that the sensitivity of the (quantitative) PCR approach to recognize clonal immune receptor rearrangements at least matches the sensitivity of Southern Blot analyses.

TCR rearrangements are detected by an approach based on the same principles as the IGH gene analysis.[11] Mostly TCRγ gene rearrangements have been studied in T cell lymphomas. Even though it is impossible to find consensus primers for all the different V and J regions, the limited repertoire of these genes allows the use of primer mixes in a multiplex PCR which identifies most rearrangements of this gene. Given the lack of D region genes in the TCRγ locus, the size variability of the amplified V gene segments is based on the imprecise V-J joining only and therefore considerably lower than in IGH V segments. Another strategy is to use consensus primers and differentially cut the amplification products with restriction enzymes or determine the differences of the individual PCR products by gradient gel electrophoresis or single-strand conformational polymorphism (SSCP) analyses.

Primer sequences have also been established for the amplification of TCRβ genes. Several primers have been developed which recognize a set of different V, D and J region genes. Even though a substantial number of cases with clonal TCRβ gene rearrangements are missed, this methods seems to be useful as an additional test for clonally expanded T cells expressing the αβ heterodimer. TCRδ PCR has widely been used for the detection of clone-specific rearrangements in acute lymphoblastic leukemia. Primers established from the unique sequences of the VDJ or VDDJ junctions of leukemia blasts are clone-specific.[12] With such a clone-specific, quantitative PCR approach a sensitivity level of one in 10^6 cells can be reached which is well suited for minimal residual disease (MRD) detection. Amplification of TCRδ gene sequences has not been performed in a large series of lymphoma cases since the TCRδ genes are frequently deleted in mature T cell neoplasms.

Consensus Vα and Jα region primers for genomic amplification of TCRα gene recombinations are not used. Reverse transcribed cellular RNA can be amplified using C region oligonucleotides and degenerate 5' end primers for the cDNAs in a so-called RT-PCR.[13] Cloning and sequencing of the amplification products or separation of the fragments on a gradient gel or by SSCP analysis allows the detection of a predominant, clonal rearrangement. The PCR procedures for TCRα gene rearrangement detection are hence more sophisticated than TCRγ/δ PCR, require cell suspensions or fresh/fresh-frozen tissue and may not be widely applicable for routine diagnostic purposes.

PCR detection of IGH and TCR rearrangements in lymphoproliferative disease

The sensitivity and specificity of the PCR approach for the detection of T and B cell clonality has been tested extensively in the past. In several large series encompassing several hundred B cell non-Hodgkin's lymphoma (NHL) specimens 70-80% of cases studied with IGH PCR revealed clonal amplification products. Serial dilution experiments of clonal with polyclonal DNA suggest a clonal detection limit of 1% or lower. False positive results seem to be exceedingly rare when cross-contamination is avoided and appropriate controls are run with the clinical samples. Even though the amount of target DNA needed for successful amplification is low, it is advisable to use DNA concentrations corresponding to more than a hundred B cells. This helps to avoid preferential primer binding which may lead to a pseudoclonal amplification result with the IGH primers.[14] Immunohistochemical analysis of the specimen which should always be done prior to molecular analysis, provides an estimate of the proportions of T and B lymphocyte populations in the lymphoma tissue. Additional use of primers directed against the FR2 region of the IGH variable gene results in a higher detection rate of clonality (greater than 85%) in B-cell lymphoma cases than the employment of FR3-region primers only (about 75%). This is explained by a lack of primer binding due to deletions and extensive hypermutations occuring predominantly in the FR3 and less so in the FR2 region sequences. Alternatively, the consensus V region primers may not anneal to some rare or unknown VH genes participating in the IGH rearrangement. With FR3 primers, low-grade B-NHL with the exception of centroblastic/centrocytic lymphomas and those of mucosa-associated lymphoid tissue (MALT) nearly always have detectable clonal PCR products. The detection rate in high-grade B cell lymphomas ranges from 75 to more than 80%. The percentage of positive cases tends to be lower among centroblastic lymphomas (60 -70%) but not in large cell anaplastic lymphomas of B-type (B-LCAL) [Griesser, unpub-

lished]. Using both FR3/FR4 and FR2/FR4 primer sets we also detected clonal IGH rearrangements in 2 of 12 cases of nodular sclerosing and 4 of 8 mixed cellularity Hodgkin's disease (HD) in a recent study.[15] Among 7 samples of nodular lymphocyte predominant HD, only one case with a high-grade B cell lymphoma component showed clonal IGH rearrangement. Detection of B-cell clonality thus helps to distinguish B-NHL from lymphocyte predominant HD and favors a B-NHL over nodular sclerosing HD, but it is certainly not a reliable criterion in the differential diagnosis of mixed cellularity HD and T-cell rich B-cell lymphoma (TCRBL). The finding of more than three clonal PCR products indicates that more than one B-cell clone is present. Such biclonal or oligoclonal rearrangements are detectable in rare cases of follicular center cell-derived lymphomas and acute lymphoblastic leukemias (ALL).[16] The majority of B-NHL, however, has only one predominant clonal band in routine PCR examinations detectable with simple size selection procedures on agarose or non-denaturing polyacrylamide gels. Amplification of short PCR products with FR3 and FR2 primers is as effective with DNA extracted from well-preserved formalin-fixed tissues as with cellular DNA from fresh/fresh frozen samples. Even though clonality is detected in nearly all B-cell lymphoma cases by Southern Blot but missed by PCR in 10-20% of cases, we have seen rare cases that were PCR-positive and Southern Blot negative.[16] Primers established from the unique sequences of the VDJ junctions of leukemic lymphoma cells are clone-specific. With such a allele-specific primers PCR detection of MRD reaches a sensitivity level of one in 10^6 cells.[17]

TCRγ PCR findings have been reported for a few hundred cases of T-NHL. Clonality is found in more than 85% of the lymphoma samples with no false-positive results. A combined investigation of T cell neoplasms with primers for both, TCRγ and TCRβ, may lead to even superior results. Our studies on 48 LCAL cases has shown that TCRγ-PCR helps to identify T lineage in more than half of the tumor cell populations lacking surface expression of T cell markers in routinely-fixed samples.[15,18] Investigations of ALL samples were mostly performed for MRD detection. A positive result in bone marrow-derived DNA seems to have clinical relevance in predicting relapse. Detection of circulating tumor cells, however, appears clinically insignificant for lymphoma patients in otherwise complete remission.[19]

Biclonal or oligoclonal TCRg rearrangements are sometimes detected by PCR in angioimmunoblastic lymphadenopathy (AIL)-like T-cell lymphomas and rarely in cutaneous T-cell lymphomas. A considerable proportion of $CD3^-$, $CD56^+$ natural killer cell-like large granular lymphocyte (NK-LGL) leukemias/lymphomas lack TCR rearrangements by PCR as well as Southern blot studies. At least some cases from female patients can be studied for clonality with alternative PCR methods, such as analyses for methylation patterns of the human androgen receptor genes.[20]

Unexpected results in rearrangement analyses by PCR

Failure to detect clonal rearrangements can have technical reasons. A clonal cell population may not be well represented in the specimen used for DNA extraction; the DNA may be severely degraded and not even suitable for PCR amplification; DNA preparations from routinely processed tissue may contain PCR inhibitors. In the latter two instances PCR amplification of ubiquitous genes will fail in control experiments. Some AIL-type T-NHL and stage I and II mycosis fungoides may not contain molecularly detectable T cell clones. The absence of a clonal gene rearrangement in the presence of a histologic pic-

ture typical for malignant lymphoma is diagnostically irrelevant. Examinations can be repeated in subsequent biopsies or specimens taken from a different site if molecular genetical support of the diagnosis is crucial.

Complete IGH rearrangements are a feature of B-cells, complete TCR rearrangements highly characteristic of T- cells. Cross-lineage rearrangements detected in Southern blot experiments mostly represent incomplete erroneous immune receptor gene rearrangements or, more rarely, translocation events involving the IGH or TCR gene locus. The detection of clonal IGH rearrangements by PCR has an advantage over the Southern blot procedure since only complete VDJ rearrangements will be amplified enzymatically, but not incomplete rearrangements or rearrangements resulting from chromosomal translocations involving the IGH gene locus. Incomplete cross-lineage IGH rearrangements in neoplastic T cells, which are detectable in Southern Blot analyses, will not be amplified by PCR which makes lineage assignment to the B cell series straightforward. However, complete TCR cross-lineage rearrangements may be detectable by PCR in cases of common ALL and pre-B lymphoblastic lymphoma. In addition to the IG genes, several TCR loci frequently undergo rearrangement and these illegitimate rearrangements may be complete. This renders genotypic lineage determination in such cases by Southern blot procedures or PCR unreliable.

In AIL-type T cell lymphomas IGH rearrangements are not infrequently detected by PCR and are likely to be due to the existence of a true B cell clone coexisting with the neoplastic T cell population. Recently we found some cases among TCRBL samples with clonal IGH as well as TCRγ rearrangement in PCR studies. Morphology and immunohistochemical results suggested that these cases were indeed composite lymphomas with a high-grade B-cell and a low-grade T-cell component.[21,22]

The occurrence of clonal TCR rearrangements is diagnostically challenging in lymphoproliferative T cell disorders which are clinically considered non-malignant. Activated T cell clones especially in lymphoproliferations of the skin may expand to a point where they become detectable by rearrangement studies but remain localized and controlled by the immune system. Clonal cutaneous lymphoproliferations such as lymphomatoid papulosis (LYP) lesions, however, may coincide with or transform into a malignant T cell lymphoma. A clonal TCR rearrangement is of limited value for the differential diagnosis between LYP and a cutaneous LCAL. T lymphocyte clones may also expand due to persistent antigen exposure. These lymphoproliferations are mainly oligoclonal but it was shown that single reactive cytotoxic T cell clones may be detectable in healthy, elderly individuals.[23]

Molecular genetical studies require a dedicated laboratory with sufficient volume of samples and significant test experience. Failure to detect clonality, or the finding of unexpected or illegitimate TCR rearrangements is rare under these circumstances. Diagnostic problems arise particularly when molecular results are not correlated with histological and immunophenotypical findings. Problems with unexpected results are mostly avoided when molecular studies in a routine laboratory setting are restricted to diagnostically challenging cases and the search for minimal disease.

Common chromosomal translocations in malignant lymphomas

Different from stepwise rearrangement processes during B cell ontogeny, chromosomal translocations involving bcl-2, bcl-1, bcl-6 and c-myc can be viewed as an accident during B cell development and activation. They most frequently involve the IGH gene

locus on chromosome 14q32 and sometimes the IGL gene loci. In the majority of cases involving bcl-1 and bcl-2, translocations can be pinpointed to an early stage of B cell differentiation before the heavy chain genes have completed their rearrangement. Translocations of the bcl-6 gene can involve a number of chromosomes besides those harboring immune receptor genes. These chromosomal abnormalities not only serve as clonal markers but also aid in the classification of lymphoma subtypes.[24] Bcl-2/JH recombination is highly characteristic of germinal center cell derived lymphomas and bcl-1/JH recombination is mainly detected in mantle zone cell-derived lymphomas. The t(11;14) is not observed together with the t(14;18) translocation and bcl-2 rearrangements are undetectable in mantle cell lymphomas. The finding of one of these translocations is therefore useful in the differential diagnosis of follicular lymphomas and mantle cell lymphomas with a nodular growth pattern. Bcl-1 rearrangement analyses also aid in the identification of blastic variants of mantle cell lymphoma.[25] Among the high-grade NHL, c-myc rearrangements are a constant feature in Burkitt's lymphoma and bcl-6 translocation is frequently detected in centroblastic lymphomas. The only common and consistent translocation in T-lineage lymphomas results in the NPM/alk fusion which is almost exclusively found in T- LCAL.

Molecular probes are being generated through isolation and sequence analysis of regions flanking the chromosomal breakpoints. These probes are useful for the detection of chromosomal translocations in Southern blot analyses if the breakpoints are clustered and not spread out over a large chromosomal region. After identification and cloning of specific chromosomal breaks their sequences can be analyzed and tumor-specific PCR primers flanking the breakpoint be designed. This PCR approach is highly suitable for molecular follow-up studies in a particular patient since amplification products are only generated from cellular DNA carrying this translocation. Fusion gene transcripts from reciprocal chromosomal translocations are detectable by PCR amplification of cDNA (RT-PCR).

Mechanisms and methods

The bcl-2 oncogene is located on chromosome 18q21. The breakpoints on chromosome 18 in the t(14;18) translocation, which is very characteristic of germinal center cell derived lymphomas irrespective of the growth pattern and blast cell content, are clustered around two regions.[26,27] In about 70% of cases the breakpoint occurs in the major breakpoint region (MBR) which is located in the 3' untranslated region of bcl-2 exon III. In most of the remaining cases it occurs in the minor cluster region (mcr) located more than 20 kb 3' of the mbr. The breakpoint on chromosome 14q32 is found within the JH gene cluster. Translocation of the bcl-2 gene is found rarely in chronic B-lymphocytic leukemia involving predominantly IGL gene loci rather than the IGH gene locus; the breaks on chromosome 18 tend to be outside and usually 5' of the mbr/mcr. The bcl-2α and bcl-2β proteins are involved in cell death regulation and have been shown to block apoptosis. Bcl-2 overexpression as a result of the translocation t(14;18) may lead to inadequate survival of B cells and render them susceptible to additional genetic aberrations.

The bcl-1 locus resides on chromosome 11q13 and is involved in the translocation t(11;14). The chromosome 11 sequences join within the J region cluster of the IGH gene locus on chromosome 14q32. Again, one predominant breakpoint region is described (major translocation cluster, mtc) on chromosome 11.[28] At least two more sites have been identified where breakpoints occur within chromosome 11q13. Aside from occasional

cases of B-CLL and multiple myelomas this translocation is characteristic of mantle zone derived B-cell lymphomas where it is found in about 50% of the mantle cell lymphoma cases with mtc probes. With the additional use of probes for the minor breakpoint cluster regions the percentage may be even higher than 70%. In contrast to the bcl-2 oncogene, the bcl-1 locus does not harbor an oncogene but likely is linked to a regulatory gene sequence of PRAD1, which is telomeric to the breakpoint region. The translocation leads to constitutive expression of the gene product cyclin D1 which promotes passage through the G1 phase of the cell cycle.

Translocations involving the c-myc locus on chromosome 8q24 are somewhat more complex.[29] They are a constant finding in sporadic and endemic Burkitt' lymphoma cases and most frequently involve the IGH genes in a t(8;14). Rarely the IGκ genes on chromosome 2p12 or IGλ genes on chromosome 22q11 can also be translocated to the myc locus. The breakpoint on chromosome 8 in the t(8;14) generally lies 5' or within the c-myc gene, whereas in variant translocations t(2;8) or t(8;22) involving IGL chain loci the break occurs 3' of the c-myc gene at distances up to 300 kb. Translocations t(8;14) are characteristic of Burkitt's lymphoma. Two different breakpoints occur depending on the type of Burkitt's lymphoma. In most endemic cases (eBL), the break occurs more than 20 kb upstream of the myc locus and involves either the J_H or the switch m region on chromosome 14. In most sporadic types (sBL) exon I of the c-myc gene or 5' flanking sequences are involved, and more often the switch m or switch g regions than the J region take part on chromosome 14. Translocation into the JH or JL region suggest that the translocation occurs at a pre-B cell stage whereas translocation into the IG switch sequences potentially take place throughout the B cell differentiation process. Expression of c-myc is high in proliferating cells and rapidely induced in quiescent cells on mitogenic stimuli. In addition to mediating cell proliferation, c-myc is also implicated in blocking the cellular programs of differentiation. Highly proliferating cells with a differentiation block are prone to apoptosis. Translocation of c-myc gene generally results in constitutive expression of this otherwise tightly regulated oncogene. Besides translocations, mutations in certain cluster regions of the c-myc gene have a similar effect and are detected in more than 50% of Burkitt's lymphomas.[30]

Chromosomal alterations affecting the bcl-6 gene at band 3q27 are a frequent recurrent abnormality in high-grade B-cell lymphomas.[31] Several partner chromosomes are involved including the sites of the IGH genes at 14q32, the IGk genes at 2p11 and the IGλ genes at 22q11. Bcl-6 seems to function as a transcription factor that binds specific DNA sequences and represses transcription from linked promoters.[32] High Bcl-6 expression is restricted to mature B-cells inside the germinal centers and has been shown to be a key regulator of germinal center formation and B-cell immune response. Chromosomal translocations usually disrupt the gene in and around the first exon leaving the coding domain intact. On the partner chromosome the bcl-6 coding domain is juxtaposed to a heterologous promoter resulting in a new chimeric transcript which encodes a normal bcl-6 protein. The promoter substitution prevents bcl-6 downregulation and blocks post-germinal center maturation of B-cells. Bcl-6 rearrangements are highly specific for high-grade B-NHL of centroblastic type where they are detected in 35% of cases. Additionally they are found in a small fraction of follicular lymphomas. Additionally, in about 70% of the high-grade B-NHL and 50% of follicular lymphomas the bcl-6 gene is affected by multiple, often biallelic mutations clustering within the 5' non-coding sequences.[33] It has been suggested that those large cell lym-

phomas only with bcl-6 rearrangement may represent de-novo high-grade B-NHL whereas those with bcl-6 and/or bcl-2 alterations may be secondary high-grade lymphomas with a less favorable prognosis.[34,35]

Chromosomal translocations may result in a novel fusion transcript that can be detected by RT-PCR. This is the case in LCALs with the translocation t(2;5) (p23; q35).[36] The nucleolar phosphoprotein nucleophosmin (NPM) gene on chromosome 5q35 is translocated to the anaplastic lymphoma kinase (alk) on chromosome 2p23 in about 60% of adult and over 80% of childhood LCAL of T- or 0-type.[37] The result is a chimeric protein with the amino terminus of the gene coding for NPM and the carboxy terminus coding for alk. Replacement of the 5' alk sequences switches the transcription regulation of the catalytic sequences of alk to the NPM promoter. Regulatory sequences of the NPM gene, which is transcriptionally most active before the cell entry into the S phase, may activate the alk gene. This kinase gene is physiologically not transcribed in lymphocytes. The fusion protein of the two genes could potentially phosphorylate intracellular substrates which are normally under control of lymphoid lineage-specific kinases only. This deregulation eventually triggers the malignant transformation of T-lineage cells.

PCR detection of chromosomal translocations

The molecular detection of these abnormalities is most reliably done by conventional Southern blot technique that identifies abnormal restriction fragment gel mobilities. The use for routine analysis, however, is limited since the variability of the breakpoints requires the application of several probes and DNA restriction with several enzymes. This also explains why rearrangements are less frequently detected by PCR, which is performed with a limited set of primers (since not all the sequence data for the exact breakpoint locations are available) and can only amplify short DNA stretches in routine applications. On the other hand, once a translocation is detected by the PCR approach in individual patients, it is far more sensitive as a clonal marker than the Southern Blot procedure.

Translocations t(14;18) involving the IgH gene locus and the bcl-2 gene region are detected by PCR with consensus primers for the JH region of the mbr or the mcr region.[38-40] Sensitivity and specificity are enhanced if sets of outer and inner primers are sequentially applied in the so-called nested PCR reaction. Alternatively, Southern blotting of PCR products with subsequent probing can be done using a radioactive-labeled internal oligonucleotide.

Chromosomal rearrangements of the bcl-1 gene locus have been amplified by PCR using mtc breakpoint oligonucleotides.[41,42] This approach is not reliable enough for routine diagnostic purposes since various minor breakpoint sites are also involved in mantle cell lymphomas. Designing, testing and using many different primer sets for these additional breakpoint locations is impracticable in a routine laboratory setting.

A limited number of cell lines and some Burkitt's lymphoma samples have been analyzed by PCR using primers for the switch region of the μ heavy chain gene, and sequences flanking and inside exon I of c-myc. With this approach it is possible to detect some of the translocations t(8;14) in sBL cases.[43] With new generations of Taq polymerases it has become possible to amplify sequences of more than 10 kb length. Using primers for IG constant region genes and primers flanking sequences 5' to breakpoint cluster regions it is possible to amplify a considerable proportion of those c-myc and bcl-6 translocations by long-distance (LD-) PCR that were formerly only detectable by

Southern blot.[44] However, template DNA has to be extracted from fresh/fresh-frozen tissue samples or cell suspensions and routinely-processed specimens cannot be analyzed.

The presence of chimeric mRNA from the NPM/alk fusion is detected by RT-PCR.[45] Even though RNA extracted from routinely-processed specimens is strongly degraded reverse transcribed cDNA fragments of 300 bp are readily amplifiable. This leads to the detection of fusion transcripts in 40-60% of T-LCAL cases by RT-PCR.

Advantages and drawbacks of translocation analyses by PCR

Most of the experience with the detection of translocations by PCR in malignant lymphoma has been accumulated for the t(14;18) abnormality. About 80% of the Southern blot-positive follicular lymphomas are PCR positive suggesting that PCR analysis for bcl-2/IGH translocation is a good molecular marker for the t(14;18) abnormality in follicular lymphomas. The PCR technique is more sensitive in the detection of qualitative rather than quantitative abnormalities such as clonal immune receptor gene rearrangement on a background of polyclonal cells. It is therefore well suited for analysis of minimal disease in lymphomas carrying the t(14;18). Reports about positive PCR results in follicular hyperplasia should be considered when looking for MRD.[46,47] The (overly) high sensitivity may be achieved by using high amounts of template DNA (> 1μg) in the reaction and very efficient primers and cycling conditions. Similarly, the high degree of sensitivity may also be responsible for some reports about surprisingly frequent detection of t(14;18) PCR products in Hodgkin's lymphomas. False-positive PCR results have to be avoided in MRD detection. DNA from the original tumor should be run in parallel when looking for minimal infiltration. Identity of the amplification products is confirmed by identical size on polyacrylamide gels and/or by sequencing. A quantitative PCR approach would also help to establish the relative number of cells in the sample which contain the rearrangement, but this approach usually goes beyond routine analysis.

It is even better not only to detect chromosomal abnormalities with high sensitivity but also to identify the cells carrying the genetic abnormality. This is possible using another fast molecular diagnostic approach: the detection of translocations by non-radioactive, usually fluorescence-based, in-situ hybridization of chromosomes (FISH). The probes detect chromosomal DNA either from metaphase spreads or non-mitotic cells.[48] The latter so-called interphase cytogenetic analysis can be done even on previously stained cells. Simultaneous immunophenotyping of the cells helps to focus the interpretation of the results on the tumor cell population.[49] Genomic DNA probes flanking the breakpoint on both sides and derived from different chromosomes are labelled with two different fluorochromes.[50] The two signals would be located on different chromosomes in a metaphase spread or found far apart in interphase cells if the translocation is absent. Joining of genetic material from the two chromosomal localizations, in contrast, leads to a doublet fluorescence signal after hybridization. Results from FISH analysis are usually available within two days, which favourably compares to the Southern blot procedures. When more translocation-specific probes become available, molecular cytogenetics promises to be a fast and simple technique for the detection of nonrandom chromosomal abnormalities. Though less sensitive than PCR detection, FISH is potentially of higher specificity in tracing single tumor cells carrying a specific anomaly. The hybridization efficiency of FISH probes in sections from archival material depends on the fixative and the age of the paraffin blocks. A negative

result in these instances is diagnostically not helpful.

Conclusions

Molecular genetic analysis has become routine for laboratories involved in lymphoma diagnosis. If precautions against sample contamination are taken, the PCR technique generates reliable results within one or two days when applied to routinely processed biopsy samples and cytology specimens containing only a hundred cells. Standard PCR procedures include assessment of lymphocyte clonality using primers for TCRγ and IGH genes as well as the search for a t(14;18) in B cell neoplasms of putative follicular center cell origin. If the results are unsatisfactory, additional primer combinations may be used and/or fresh/fresh-frozen tissue has to be used for DNA studies with Southern Blot techniques. Since no primer sequences suitable for PCR amplification of TCRα and IGL rearrangements are available, these tests are performed by Southern blotting. This is also the method of choice for an in-depth analysis of chromosomal translocations involving the c-myc, bcl-1, bcl-6 or bcl-2 loci, for example in the setting of prospective clinical studies. RT-PCR is successfully applied for the detection of t(2;5) translocations in LCALs because of the fusion transcripts generated by this abnormality. Limitations of the molecular techniques are well recognized and can be handled in an experienced laboratory. The diagnostic process should begin with the cytomorphological evaluation of the specimen. Even if microscopic examination does not lead to a conclusive diagnosis, it provides a hypothesis for immunohistochemical and, ultimately, molecular genetic testing. Molecular test results have to be viewed in the context of morphology and immunohistochemistry. The detection of a clonal lymphocyte population in clinical samples is always abnormal but it should never be considered a proof of malignancy. Similarly, genomic instability may result from abnormal lymphocyte activation or destabilizing influences upon chromosomal DNA leading to translocations. Some of these translocations seem to be necessary but are not sufficient for lymphomagenesis. Thus, the detection of translocations in rare cells by a highly sensitive PCR procedure should not be taken as evidence of malignant lymphoma out of the cytomorphological context. On the other hand, a PCR result can be negative for clonality for many reasons besides the absence of a clonal lymphocyte population, such as lack of primer binding, suboptimal PCR conditions or problems related to the extraction of suitable template DNA or RNA.

Keeping the limitations in mind, immune receptor gene rearrangement studies are well suited to define lineage and clonality of a lymphoproliferation. Analyses of chromosomal translocations, in addition, provide information about the lymphoma subtype, its biologic behavior and mechanisms of lymphomagenesis. Furthermore, the high sensitivity and specificity of the PCR-based molecular tests are most helpful for monitoring lymphoma therapy, identification of MRD and early diagnosis of relapse. In an optimal laboratory setting, cytomorphologic, immunophenotypic and molecular results are interpreted jointly by individuals with expertise in all three methods in order to achieve the best diagnosis possible for the patient.

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In vivo electroporation of the urinary bladder in mice

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Cell membrane is the major obstacle to incorporation of different substances into cells. Especially in the urothelium of the mammalian urinary bladder, plasma membrane of superficial cells acts as a strong and thick barrier, preventing penetration of exogenous molecules into the cytosol. Electroporation is one of the methods which enable access of different cytochemical labels to the cytosol; so far, however, it has not been used on the urothelial cells in vivo. Therefore, the aim of this study was to determine whether in vivo electroporation of the urinary bladder is a suitable method for introduction of labels into the cytosol of urothelial cells. Labels of various molecular masses were introduced: trypan blue, TRITC-phalloidin and FITC-labelled antibody (IgG). The results demonstrated that electroporation in vivo was a suitable method for introduction of labels into the cytosol of urothelial cells and could be used as a technique for detection of intracellular molecules and studying biochemical reactions.

Key words: bladder; electroporation, trypan blue, fluorescent dyes

Introduction

Electroporation is a method used for introduction of different molecules into the cytosol *in vitro* and *in vivo*.[1,2] Exposure of cells or tissues to short intense electric pulses induces transient electropores in the membrane, which, under suitable conditions, does not affect viability of cells. Recently, electroporation has been used for drug delivery as well as gene delivery *in vivo*.[2,3] Based on preclinical studies, successful employment of electroporation for delivery of chemotherapeutic drugs such as bleomycin and cisplatin has been confirmed also in clinical trials on cancer patients.[4,5] Electroporation is widely used for *in situ* biochemical studies in cells. It has proved its efficiency for introducing molecules of different molecular weight either for labelling intracellular components or for studying biochemical pathways.[6,7]

Urothelial cell membrane is asymmetric, with specific protein uroplakins on the outer surface of the membrane. This asymmetric unit membrane protects the underlying epithelium from the toxic urine and is believed to play a role in strengthening the urothelial apical surface in order to prevent tissue rupture during bladder distension.[8] Functioning as a strengthening and a barrier, the plasma

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membrane of uroepithelial cells is highly impermeable. In order to penetrate this strong barrier, several immunocytochemical as well as electron microscopical methods were employed.[9,10] Due to its broad application electroporation could also be used for introduction of molecules into the urothelial cells. Further, this method has potential application in the treatment of urothelial tumors since introduction of chemotherapeutic drugs into the tumor cells by electric pulses (electrochemotherapy) was demonstrated to be effective. However, those tumors were not grown *in situ*, but transplanted subcutaneously into the back of the mice.[11]

The aim of this work was to determine whether *in vivo* electroporation of the urinary bladder was a suitable method to introduce molecules of various molecular masses into the cytosol of urothelial cells. If electroporation proves suitable in the present study, it could also be used for cytochemical studies, i.e. the introduction of labels into the cells to detect molecular constituents as well as chemotherapeutic drugs.

Material and methods

Mice

In the experiments, an inbred strain of NIH mice was used, purchased from Krka d.d. (Novo Mesto, Slovenia). Mice were maintained at a constant room temperature (24 °C) and natural day/night light cycle, in a conventional animal colony. Before the experiments, the mice were subjected to an adaptation period of at least 10 days.

In vivo electroporation

Adult female mice were anesthetised with an intraperitoneal injection of a mixture of Ketanest 150µg, Rumpun 10µg and atropine 0.1µg per 1g of body weight (Parke-Davis GMBH). Abdomen of the mice was opened and the urinary bladder exposed. After rinsing the bladder with saline, 0.2 ml of the label was injected intravesically. As labels, molecules of various molecular masses were used: 10 mg/ml trypan blue (MW 961) (Sigma), 7.8 µg/ml TRITC-phalloidin (MW 1305) (Sigma) and 10µg/ml FITC-anti rabbit IgG (155 kDa) (Sigma).

Application of electric pulses was performed as described previously.[12] Briefly, electric pulses were delivered by two flat parallel stainless-steel electrodes, 8 mm apart from each other (two stainless-steel strips, length 35 mm, width 7 mm with rounded edges) to the exposed bladder. Eight square wave electric pulses of 720 V or 1040 V amplitude, pulse width 100 µs and repetition frequency 1 Hz, were generated by an electropulsator Jouan GHT 1287 (Jouan, France).

Electron microscopy

Ten minutes after the application of electric pulses, the bladders were fixed with 4% paraform aldehyde and 2% glutaraldehyde in cacodylate buffer, postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer, preembedding stained with uranyl acetate, dehydrated and embedded in Epon. Ultrathin sections were stained with lead citrate and examined in a Jeol 100 CX electron microscope.

Light microscopy

Ten minutes after the application of electric pulses, the bladders were fixed in 4% formaldehyde. After rinsing, the tissue was frozen and sectioned with a cryotom. The sections mounted in a Slow fade mounting solution (Molecular Probes) were examined under a conventional or fluorescent microscope (Laborlux Leiz).

Results

Electroporation

Light microscopy examination of tissue in the bladder exposed to 8 electric pulses of amplitude 1040 V established that large areas of the urothelium were detached from the basal lamina. Undetached urothelial cells were loosely connected to each other (Figure 1).

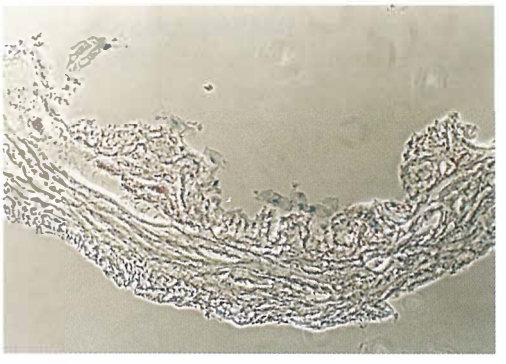

Figure 1. The bladder exposed to electric pulses at an amplitude of 1040 V. Large areas of the urothelium were detached from the basal lamina, 32x.

It was obvious that on the ultrastructural level the most severe changes took place at the layer of basal cells. Many of these cells were split into half, parallelly to the basal lamina (Figure 2). In undetached superficial urothelial cells, facing the bladder lumen, the membranes of fusiform vesicles were torn at the edges where the normal membrane linked two asymmetric unit membrane plaques.

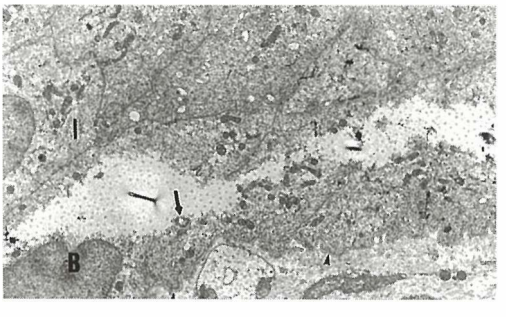

Figure 2. In the tissue, electroporated at 1040 V, tearing of basal cells (B) was evident (↑). In intermediate cells (I) no morphological changes were seen. (V) basal lamina, 2600x.

In order to avoid damage to the cells by electroporation, lower amplitudes of electric pulses were tested in the second set of experiments. No morphological changes were observed in the bladder urothelium when the bladder was exposed to electric pulses of amplitude 720 V (Figure 3). Therefore, all the following experiments were performed at this amplitude of electric pulses.

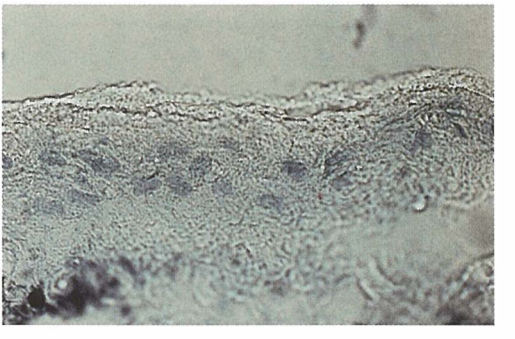

Figure 3. Trypan blue staining of urothelial nuclei (↑). The bladder was exposed to the electric pulses, at an amplitude of 720 V, 128x.

Labelling of the urothelial cells

In the preliminary experiments of this study, it was proved that none of the labels that we used could penetrate the urothelial cells.

After the application of electric pulses to the bladder (amplitude 720 V), trypan blue (MW 961) dye entered the urothelial cells and was evenly distributed over the whole urothelium. Nuclei of all the three epithelial layers were stained intensively. In the connective tissue only sporadic nuclei were stained (Figure 3). TRITC-phalloidin (MW 1305) entered only the superficial urothelial cells and labelled the actin filaments at the basolateral membranes. The actins in the intermediary and basal layer of cells were not stained (Figure 4). Loading of cells with FITC-IgG (MW

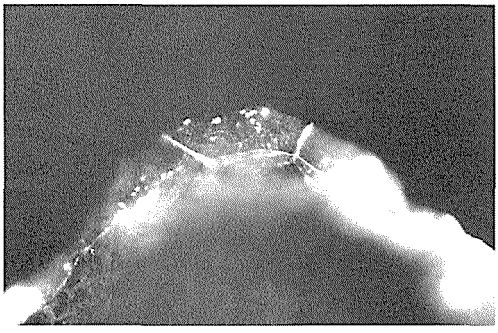

Figure 4. Phalloidin labelling of actin observed mainly at the cell borders of superficial cells. The bladder was exposed to electric pulses at an amplitude of 720 V, 128x.

155 kDa) was successful in approximately 2/3 of superficial cells. The fluorescence was distributed equally in heavy loaded cells. No labelling was found in the cells beneath the superficial ones (Figure 5).

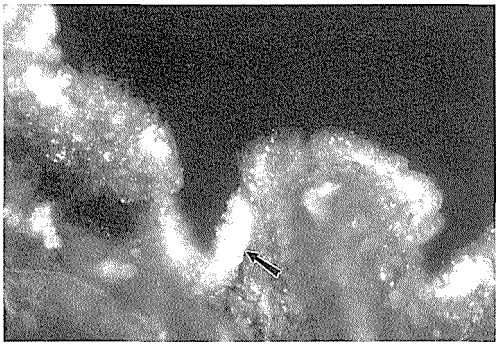

Figure 5. FITC- IgG entered most superficial cells (↑) after the application of electric pulses at an amplitude of 720 V to the bladder, 128x.

Discussion

In this study labels of various molecular masses, trypan blue, TRITC-phalloidin and FITC-labelled antibody (IgG), were introduced into the urothelial cells by electroporation. Our results demonstrate that electroporation enables penetration of the label through a thickened plasma membranes and could therefore be used as an additional technique for the detection of molecular constituents inside the cells and studying biochemical reactions.

Electroporation *in vitro* has already proved its usefulness for labelling intracellular constituents with specific labels which can not enter intact cells.[1] For example, actin network and intermediate filaments were visualized after the introduction of FITC-phalloidin and FITC-vimentin by electroporation of human gingival fibroblasts.[6] Our results demonstrate that electroporation is also a suitable method for introduction of labels into the urothelial cells *in vivo.*

All the labels entered the superficial cells at electric pulses amplitude 720 V without visible changes in cell morphology. The effectiveness of label penetration depended on their molecular masses. The smallest label trypan blue (MW = 961) entered all the urothelial cells, but was mainly prevented from staining nuclei of fibroblasts in the lamina propria. In such cases, basal lamina may act as a barrier. Phalloidin, a molecule that is much larger (MW 1305), labelled the actin filaments only in superficial cells. The label was bound to actin filaments only in basolateral region. Such pattern of actin filaments distribution is also known in urothelial cells which have not been electroporated. The results show that phalloidin has not entered intermediate and basal cells. We can not exclude the possibility that actin from superficial cells has bound most of the phalloidin and so reduced the concentration of phalloidin passing through this dense layer of actin towards intermediate cells, below the sensitivity of fluorescence microscope. Loading of most superficial cells with FITC-IgG provides the possibility of using electric pulses as a new method for labelling cell's constituents in vivo with labels that have a high molecular weight.

In conclusion, this study expands the area of application of electric pulses *in vivo* from electrogene therapy,[3] electrochemothera-

py,[2,4,5,12] transdermal drug delivery[1,3] to *in situ* studies of cellular constituents and biochemical pathways in the bladder.

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MDP desmuramyl analogue LK-404 protects bone marrow and spleen cells from cyclophosphamide induced apoptosis

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In this article, we present the data on induction of apoptosis in mouse bone marrow cells and spleen cells after treatment with different concentrations of cytostatic cyclophosphamide in vivo and in vitro. Increasing apoptosis rate of the cells was observed with the increasing concentration of cyclophosphamide, and with the prolongation of incubation time after in vivo as well as in vitro administration of the drug. When apoptosis inducing activity was established, the immunomodulatory and feasible protective effect of desmuramyl analogue of MDP (LK-404) against cyclophosphamide induced apoptosis in mouse bone marrow and spleen cells was studied. Cultivating the cells with cyclophosphamide and LK-404 simultaneously revealed the same apoptosis rate as cultivating with cytostatic only. Treatment of cells with LK-404 prior to treatment with cyclophosphamide decreased apoptosis of bone marrow and spleen cells which suggests potential protective role of LK-404 against cyclophosphamide induced apoptosis.

Key words: apoptosis, bone marrow, spleen, cytostatic, immunomodulator

Introduction

Apoptosis is a genetically controlled process of cell death.[1-5] It has significant value as counter-weight to cell division and proliferation, thus keeping the number of cells in tissue constant.[6-9] Apoptotic cells present unique changes of DNA molecule. Apoptotic process occurs spontaneously, but can also be induced. Important inducers of programmed cell death are deduction of nutrients, regulatory molecules as for instance cytokines, hormones and growth factors, switching on of several distinctive genes, treatment with radiation or with cytostatics.[10,11] Abnormalities in the process of apoptosis may have a large influence on beginning and development of diseases like cancer, viral infections, autoimmune disease and central nervous system disease.[12-18]

Apoptotic cell death can be detected by several methods.[19] In the presented experiments, the apoptosis rate was determined using a specific ELISA (Boehringer Mannheim, Germany) detecting apoptosis specific

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DNA fragments with enzyme labelled antibodies.

Cyclophosphamide is a cytostatic drug widely used in anti-tumor therapy. It has cytotoxic effect in all phases of the cell cycle, although it has stronger influence on activated or already dividing cells. Cyclophosphamide is a DNA-alkylating drug able to cross-link DNA chains, thus causing cell death.[20] It is generally known that also the cells of the immune system die as a side effect of such tumor treatment. The reduction in number of immune cells results in a decreased immune response which can lead to severe and more frequent infections.

Apoptosis in mouse marrow and spleen cells was studied in described experiments using cyclophosphamide as the inductor of cell death . [21,22] The aim of the study was to examine the apoptosis of bone marrow and spleen immune cells as an inducible process which can be stopped by appropriate treatment. It would be well appreciated, if selective reduction of apoptosis of immune cells and potentiation of toxicity for the tumor cells could be achieved. Using immunomodulating MDP derivatives (N-acetyl-L-alanyl-D-isoglutamine) with hemopoiesis restoring activity may be effective.[23-25] Therefore the attempt was done to restore the cyclophosphamide induced apoptosis in bone marrow and spleen cells with LK-404 one of MDP analogues. LK-404 (N-(7-oxododekanoyl)-L-alanine-D-isoglutamine) an desmuramyl analogue synthesized at Faculty of Pharmecy, University of Ljubljana, in cooperation with Lek d.d., it is in opposite to original MDP molecule without disadvantageous pyrogenicity, and less toxic. The potential protective effect of LK-404 against cyclophosphamide induced apoptosis was studied using LK-404 simultaneously and consecutively to treatment with cyclophosphamide.

Materials and methods

Animals and the preparation of cells

Han NmRi mice were used in all the described experiments. The animals were provided by Lek Research and Development Animal Care, Ljubljana, Slovenia. After delivery from Animal Care they were kept under standard conditions in our facilities. The mice, we used as a source of bone marrow and spleen cells, were at the beginning of the experiment, sacrificed with an ether overdose anesthesia. The femurs and spleens were isolated. The epiphyses were cut off the femur and 5 ml RPMI 1640 Medium (Sigma Chemicals; St. Louis, USA) was squirted through the bone. Washed out cells were sucked into the syringe again and then flushed to the wall of Petri dish. Spleen cells were isolated from the spleen using sintered microscope slide glass plates and afterwards suspended in growth medium RPMI 1640. Contaminating erythrocytes were removed from both cell suspensions by adding 2 ml of 0.85% TRIS buffered ammonium chloride (NH_4Cl). The remaining mononuclear cells were washed three times with MEM medium (Sigma Chemical Co., St.Louis, USA). Cells were suspended (1:10) in trypan blue (0.1%) and then counted in haemocytometer. A concentration of $5x10^5$ cells per ml was prepared for the experiment.

Apoptosis induction

Lyophilized cyclophosphamide (Endoxan, Asta Medica AG Frankfurt, Germany) was dissolved in sterile bidistilled water and solutions giving concetrations of 6.25, 12.5, 25, 50 and 100 mg of cyclophosphamide per kg of mouse weight were prepared.

In *in vivo* experiments, 200 μL of cyclophosphamide at a dose of 50 mg/kg of mouse body weight or 100 mg/kg of mouse body

weight was injected intraperitoneally. Treatment was repeated on days one, four and six. On the seventh day of experiment, the mice were sacrificed. The control group of mice was injected with sterile physiological saline instead of with cyclophosphamide at the same days.

In *in vitro* experiments, cyclophosphamide was used in appropriate equivalents at concentrations of 6.25 mg/kg, 12.5 mg/kg, 25 mg/kg and 50 mg/kg of mouse weight. The cell samples were taken 5, 10, 15, 30, 60 and 90 minutes after the addition of cyclophosphamide to the cells used in the experiment.

LK-404

The compound LK-404 was prepared in RPMI 1640 at concentrations of 0.525 μM, 5.25 μM and 52.5 μM and then used in the experiments described below.

In first series of experiments, isolated bone marrow and spleen cells were treated with LK-404 and cyclophosphamide simultaneously. Aliquoted samples were taken for testing the apoptosis 5, 10, 15, 30, 60 and 90 minutes after incubation. All three concentrations of LK-404 were tested.

In the second series of experiments, consecutive applications of LK-404 and cyclophosphamide were studied. The cells were first incubated at all three concentrations of LK-404 for 90 minutes. Ninety minutes after the beginning of the experiment, a 25 mg/kg of cyclophosphamide was added to each of the samples. Aliquots were taken for testing 5, 10, 15, 30, 60 and 90 minutes after the cyclophosphamide addition.

Cell death detection ELISA

The apoptosis rate was determined using Cell Death Detection ELISA (Boehringer Mannheim, Germany). The assay is based on the quantitative sandwich-enzyme-immunoassay principle using monoclonal antibodies directed against DNA and histones, respectively. This permits a specific determination of mono and oligonucleosomes in the cytoplasmic fraction of cell lysates. In the first step, anti-histone antibody is fixed to the wall and the bottom of the microtiter plate module. During the second step, the nucleosomes contained in the sample bind, via their histone components, to the immobilized anti-histone antibody. In the third step, anti-DNA-peroxidase labeled antibody (POD) reacts with the DNA part of the nucleosome. The amount of peroxidase retained in the immuncomplex is determined spectrophotometrically with ABTS (2,2'-azino-di-(3-ethylbenzthiazoline sulfonate)) as substrate.

Design of the experiments performed

Treatment of bone marrow and spleen cells with cyclophosphamide in vivo: 9 mice were divided into 3 subgroups. Three mice from the first subgroup were injected with 200 μl of cyclophosphamide at a dose of 100 mg/kg, three from the second subgroup with 200 μl of cyclophosphamide at a dose of 50 mg/kg, and the remaining three with 200 μl of physiological saline on the first, fourth and sixth day. The mice were sacrificed on the seventh day. Bone marrow cells and spleen cells were isolated and divided into two portions. Apoptosis of the cells from the first aliquot was measured the same day. The second aliquot was first incubated for 24 hours at room temperature; the apoptosis was measured on the second day.

Treatment of bone marrow and spleen cells with cyclophosphamide in vitro: Isolated bone marrow and spleen cells were treated with the following concentrations of cyclophosphamide: 25 or 50 mg/kg. The sample treated with RPMI 1640 alone instead of with cyclophosphamide; was regarded as a

control sample. The samples were incubated for 15, 30, 60 or 90 minutes. Aliquots were taken at indicated times. The number of live cells was determined and amount of apoptosis measured in each of the samples at each time point.

Simultaneous application of LK-404 and cyclophosphamide to bone marrow and spleen cells in vitro: Bone marrow and spleen cells were isolated. Cyclophosphamide at a concentration of 25 mg/kg and LK-404 at concentrations of 0.525 μM, 5.25 μM or 52.5 μM were added to cell suspensions. The first control sample was incubated only with 25 mg/kg cyclophosphamide, whereas the second one only with medium RPMI 1640. Aliquots for testing were taken 5, 10, 15, 30, 60 and 90 minutes after the beginning of the experiment and the amount of apoptosis was measured.

Consecutive application of LK-404 and cyclophosphamide to bone marrow and spleen cells in vitro: Bone marrow and spleen cells were isolated and incubated with LK-404 at concentrations of 0.525 μM, 5.25 μM or 52.5 μM. After 90 minutes, a 25 mg/kg of cyclophosphamide was added to each of the samples. Aliquots were taken for testing 5, 10, 15, 30, 60 and 90 minutes after adding the cyclophosphamide.

Two samples of cells were prepared without LK-404 and, after 90 minutes of incubation, the cells of the first sample were treated with 25 mg/kg of cyclophosphamide. The cells of the second sample were treated neither with LK-404 nor with cyclophosphamide. Aliquots for testing were taken after 5, 10, 15, 30, 60 and 90 minutes.

Statistical analysis

Student's t-test was used to determine the difference between samples and $p<0.05$ was regarded as significant.

Results

Treatment of bone marrow and spleen cells with cyclophosphamide in vivo

The apoptosis of bone marrow cells and spleen cells depends on the concentration of cyclophosphamide and time of incubation. The bones from the mice treated with cyclophosphamide at a dose of 50 and 100 mg/kg contained less marrow and the weight of the spleen was reduced as compared to the control nontreated mice.

Treatment of bone marrow and spleen cells with cyclophosphamide in vitro

In previous experiment, we noticed that cyclophosphamide reduced the number of bone marrow and spleen cells during the incubation. To get a real insight in the process of apoptosis, we compared the number of bone marrow and spleen cells with the amount of apoptotic products in each of the sample. The rate of apoptosis in Figure 1 is therefore presented as a quotient between the apoptosis (O.D.) and the number of cells in the sample. Apoptosis of bone marrow and spleen cells treated with cyclophosphamide depends on the concentration of cyclophosphamide and incubation time.

Influence of simultaneous application of LK-404 and cyclophosphamide on apoptosis in bone marrow and spleen cells in vitro

Bone marrow and spleen cells were treated in four different ways. In the control group, the cells were treated with cyclophosphamide at a dose of 25 mg/kg. The first group was treated with 25 mg/kg of cyclophosphamide plus 0.525 μM of LK-404, the second with 25 mg/kg of cyclophosphamide plus 5.25 μM of LK-404, and the third with 25 mg/kg of cyclophosphamide plus 52.5 μM of LK-404. In all eight groups

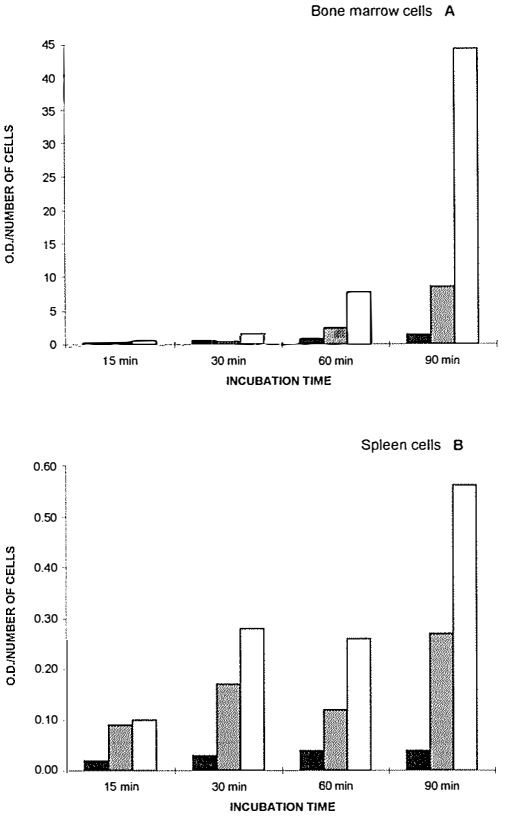

Figure 1. Panel A: Treatment of bone marrow cells with cyclophosphamide *in vitro.* In the control group (cells were not treated with cyclophosphamide), the apoptosis quotient was slowly increasing with the prolongation of incubation time. In the group where bone marrow cells were incubated with 25 mg/kg of cyclophosphamide, the apoptosis quotient was steeply increasing during the prolongation of incubation time. In the group where cells were incubated with 50 mg/kg cyclophosphamide, the apoptosis quotient was exponentially increasing with the prolongation of incubation time. **Panel B:** Treatment of spleen cells with cyclophosphamide in vitro. The apoptosis quotients of the control group (cells not treated with cyclophosphamide) were slowly increasing with incubation time. The apoptosis quotients of the group where cells were treated with 25 mg/kg of cyclophosphamide shows a tendency towards a steep increment with incubation time. The apoptosis quotients of the cells in the group where cells were treated with 50 mg/kg of cyclophosphamide increase rapidly with incubation time.

Legend: ■ control, ▨ cyclophosphamide 25 mg/kg, □ cyclophosphamide 50 mg/kg

(four groups of bone marrow cells and four groups of spleen cells), apoptosis increased by the same rate with the prolongation of incubation time without significant influence of treatment with LK-404.

Influence of consecutive application of LK-404 and cyclophosphamide on apoptosis in bone marrow and spleen cells in vitro

The rate of apoptosis of bone marrow and spleen cells in the first control sample (cells incubated in RPMI 1640 only) was slowly increasing during the incubation time due to spontaneous apoptosis of incubated bone marrow and spleen cells. In the second control sample (cells treated with cyclophosphamide after 90 minutes of incubation, and not pretraeted with LK-404) apoptosis of bone marrow and spleen cells rapidly increased after cyclophosphamide had been added, due to cyclophosphamide induced apoptosis.

The difference in apoptosis between both control samples was significant ($p=0.015$). Apoptosis in other samples (treated with LK404 prior to cyclophosphamide) increased only slowly in comparison to the control samples. The treatment of bone marrow and spleen cells with the highest concentration of LK-404 (52.5 μM) slowed down the apoptosis. The decreasing effect calculated between the second control sample (cells treated with cyclophosphamide after 90 minutes of incubation and not pre-treated with LK-404) and the sample pre-treated with 52.5 μM of LK-404 was significant ($p=0.004$ for bone marrow cells and $p=0.042$ for spleen cells). The results of these experiments are shown in Figure 2.

Discussion

The importance of apoptosis in the development and differentiation of immune cells is

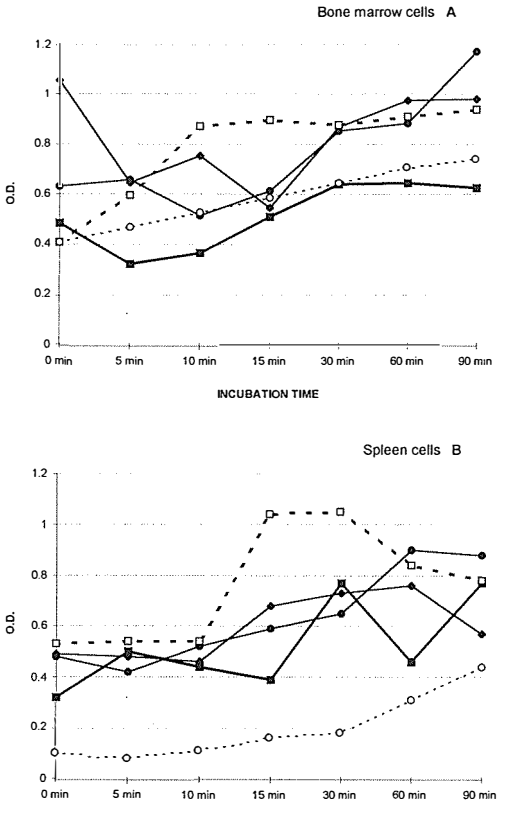

Figure 2. Panels A and B: Influence of consecutive applications of LK-404 and cyclophosphamide on apoptosis in bone marrow and spleen cells in vitro. Apoptosis of bone marrow (A) and spleen (B) cells incubated with RPMI 1640 only (- - o - -) was slowly increasing with the prolongation of incubation time. After addition of cyclophosphamide in the second control sample (- - □ - -) (cells treated with cyclophosphamide after 90 minutes, and not pre-treated with LK-404) the apoptosis increased steeply. Apoptosis in other samples (of both types of cells) treated with 0.525 μM (--●--), 5.25 μM (—◆—), and 52.5 μM LK-404 (--■--) prior to cyclophosphamide addition, decreased in comparison to the second control sample (- - □ - -) of bone marrow and spleen cells. The most effective was pre-treatment with 52.5 μM LK-404 (--■--).

not doubtful any more. At present, the focal question concerning the immune system is the involvement of apoptosis in regulation and functioning of the immune cells.[5,10, 11,21,22] However, the immune response is a self-limiting process under the influence of antigen burden, stability of the antigenic structure, activity of macrophage phagocytic system, antigenicity, capability of the antigen presentation and recognition, function of B and T cells and competence of effector functions of antibodies and cytotoxic lymphoid cells, it may also be limited by induction of apoptosis. Interference with apoptosis leaving cells alive for longer period of time, as determined by the genetically determined cell life span, would give an interesting tool to manipulate disease which depends also on immune system activity.[15,18] In malignant disease treated with cytostatics, immune cells are not prone to respond effectively. The consequences are oportunistic infections which may be the cause of death of the patient. Our experimental approach to study the importance of apoptosis in regulation of immune system function is a copy of the treatment of patients with alkylating drugs. Cyclophosphamide treatment is accompanied by a decreased function of immune system and followed by increased susceptibility to infection.[20]

Protection of the immune cells against cytostatics or adjuving depressed lymphocyte function would be of great value. MDP molecule, an important component of the peptidoglycan, has been proved to have an immunoadjuvant activity. Unfortunately, the original MDP molecule is highly pyrogenic and a good inducer of autoimmunity and as such of no use as an immuno-adjuvant. By changing the structure of MDP molecule, analogues may be prepared with a preserved adjuvant activity with no unpleasant side effects.[26] Desmuramyl analogues of MDP are able to increase the functioning of the immune cells.[27] The exact way how they do it is unknown. It would be therefore interesting to know whether such preparations would be able to interfere with the process of apoptosis.

For the induction of apoptosis we applied the widely used cyclophosphamide, a cytostatic with not yet describet apoptosis activity.

We were able to induce apoptosis with cyclophosphamide as presented in the results section. Apoptotic function of cyclophosphamide was time and dose dependent. Therefore, in patients treated with cyclophosphamide, it is expected that at least some of the immune cells will die because of the induced apoptosis. This was proved to be true in our experimental model of mice. *In vivo* treatment with cyclophosphamide resulted in increased apoptosis rate in bone marrow and spleen cells. The same was demonstrated also *in vitro* when the isolated cells were treated with cyclophosphamide. Isolated immune cells fall to apoptosis spontaneously, however, the process itself is forced by cyclophosphamide treatment.

To study apoptosis protective effect of MDP analogues (potent drugs for restoring mielosuppression) bone marrow and spleen cells were treated with MDP desmuramyl derivative LK-404. It was shown that LK-404 did not substantially increase spontaneous apoptosis at doses proposed to have an immunoadjuvant activity. Therefore, MDP treated cells were exposed to cyclophosphamide. To our surprise, apoptosis of LK-404 treated cells wasdose and time dependent and cells were less apoptotic than those treated with cyclphosphamide alone. The same result was achieved with bone marrow and spleen cells. The extent of apoptosis inhibition was greater when bone marrow cells were used as the target cells, suggesting that anti-apoptotic effect of LK-404 could be advantageous in treating the patients with the affected bone marrow.[28] The mechanism how LK-404 protects cells from the apoptosis induced by cyclphosphamide is not clear.

The potential use of anti-apoptotic phenomenon of LK-404 requires further investigations. The substance could be beneficial in the treatment of patients with suppressed activity of bone marrow as the consequence of cytostatic or radiation treatment, viral infections, prolongation of the specific immune response in the extinguishing phase of immune response, treatment of the immune cells in aged (senile) organisms. A more challenging use of LK-404 could be its application in treating Alzheimer's and Parkinson's diseases.

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review

The cause of testicular cancer

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The cause of testicular cancer, like of most other cancers, is unknown, but the groups of men with higher incidence of this type of cancer are being investigated. Familial patterns of testicular cancer are reported, as well as the influence of oncogenes, particular predisposition factors of congenital anomalies of the urogenital tract, starting with the failure of the testis descent (cryptorhism). Since the incidence of cryptorhism is growing in the world, this could prove to be the main cause of the higher incidence of testicular cancer.
The atrophy of the germinal epithelium is also ascribed a special role in the incidence of testicular cancer. The impact of the oestrogen from environment, possibly affecting the spermatogenesis and probably causing higher incidence of congenital anomalies of the urogenital tract, has been lately intensively studied as well.
Social factors may prove significant, too, while physical trauma has not been determined as the cause of testicular cancer, and it could only be considered as one of the potential co-causes of the cancer.

Key words: testicular neoplasm, etiology

Introduction

Since the incidence of testicular cancer has been growing both in the World,[1,2] and in Slovenia,[3] the possible causes of this disease have been the subject of many studies. The real cause of the testicular cancer has remained unknown, but the groups of men with higher incidence are being studied.

Genetic causes

Familial testicular cancer has been debated as a potential and possibly independent risk factor.[4] Familial patterns of testicular cancer can be related to a groups of patients with higher incidence of congenital anomalies, as well as to patients with tumours in the counter-lateral testis[5] and to Down's syndrome. Such families have been reported as displaying a 6 to 10 times higher incidence of testicular cancer. The most frequent chromosomal anomalies have been looked for, those which cause malignant alteration in testicles.[6]

An international study of 100 families with a familial form of testicular cancer, carried out under the auspices of the UK Imperial Cancer Research Fund, has revealed considerable genetic heterogeneity. There is no evidence of a single genomic region accounting for the occurrence of testicular cancer, but rather of several such regions.[7] Some sole

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cytogenetic abnormalities have nevertheless been found in patients with nonseminomatous tumours.[8]

The most frequent chromosomal anomalies related to malignant alterations in testicles are still being looked for,[6,9] and in particular there are looking for oncogenes. The most frequent oncogenes detected in testicular tumours and related to oncogenesis are K-ras2, PDGFA and N-myc oncogenes.

Predisposition factors of congenital anomalies of urogenital tract

The incidence of testicular cancer is higher in patients with the anomalies of the urogenital tract, especially the failure of testis descent (cryptorhism), but also ingvinal hernia, hydrocele and hypospadias. As much as 10% of testicular neoplasm are associated with cryptorhism, in spite of the orchriopexy.[10,11] The risk factor for testicular tumours is 40 times higher with the failure of testis descent. The risk is a little lower if the operation of the cryptorhism is performed before the age of six. The risk of cancer is also high in the contralateral descended testis.[12]

Since the incidence of cryptorhism is growing in the World, this could prove to be the main cause of the higher incidence of testicular cancer.[13,14]

Patients who have had one testicular tumour are more likely to develop a contralateral tumour.[15,16] The incidence of carcinoma in situ of the contralateral testis in patients who have had one testicular tumour is about 5%, and about 50% of patients with carcinoma in situ develop invasive malignancy within 5 years.[17]

The atrophy of testis

In to the above mentioned factors, the atrophy of the germinal epithelium is supposed to be significant as well, most frequent in the pathogenesis of tumours.[18] The reports that the atrophy, as a complication after vasectomy, is a risk factor for testicular tumours, also speak in favour of this theory.[19] When a tumour is diagnosed in a testis, higher levels of FSH would represent a risk factor for a secondary tumour.[19] The incidence of testicular cancer is also higher with fertility disorders, which are closely linked with atrophy of testis.[5]

Contrary to that, Giwercman and others[20] report that the risk of carcinoma in situ of testis is not higher in moderately oligozoospermic men. Bilateral testicular biopsies were performed in 207 men of infertile couples. No carcinoma in situ were detected in any of them. Although the authors believe that there is substantial evidence of a fetal origin of carcinoma in situ, patients in whom the biopsy revealed no carcinoma should also be closely monitored. The results presented namely refer to moderately oligozoospremic men only.

Hormonal factors

The peak incidence of testicular cancer in young men suggests that gestational development and events during early infancy and puberty are important in the pathogenesis of the disease. There are two potentially significant events: the transformation of fetal germ cells into carcinoma in situ cells and later conversion of carcinoma in situ cells into fully invasive cancer. Several hypotheses suggest an endocrinological background of testicular neoplasia.[21]

Since the inhibin B, which is considerably higher in the first year of life, could not be traced in boys with anorhism, this hormone could prove to be especially significant for the occurrence of testicular cancer. After puberty, relatively higher levels of oestrogen and androgen receptors could be relevant for the etiology of testicular cancer.[13]

Estrogens probably play an important role in the etiopathogenesis of testicular cancer. This was the conclusion of the study by Nakazuma,[22] which revealed that the serum levels of estradiol and hCG were significantly above normal values both in systemic and spermatic veins of patients with nonseminoma germ cell tumours. Higher levels of estrogens in the serum could, on the other hand, be the consequence of the tumour and not its cause, as the production of hCG is the consequence of the growth of the germ cell tumours.[23]

The oestrogen in the environment are considered to be the most important cause of the low quality of sperm in the last 20 years,[24] and they are also blamed for a higher incidence of innate malformations and possibly for the incidence of testicular cancer.

Women, who were on estrogen diethylstilbestrol during pregnancy, have been studied. Since the drug has estrogenic effect, it was presumed a possible cause of testicular cancer. But a detailed review of the effect of the in-utero exposure to diethylstilbestrol showed only an adverse effect on the reproductive tract in male progeny.[25] Tests on animals showed that while prenatal estrogen exposure does induce more severe and earlier testicular abnormalities, manifesting themselves as regressive changes in the germinal epithelium and Sertoli's cells, atrophied seminiferous tubules and dysplasia of the rete testis epithelium, the presence of neoplasm has never been confirmed.[26]

The impact of diethylstilbestol, taken by pregnant women, on the incidence of vaginal cancer in their daughters has been confirmed, and the estrogens from the environment are likely responsible for a higher incidence of breast cancer. The hypothesis of these estrogens promoting testicular cancer is still viable,[27,28] and the development of serum markers determining the exposure of an individual to estrogens in the environment is very important.[29] The investigation into the impact of estrogens on the incidence of testicular cancer is very insensitive. Oestrogen has been related to the lower quality of sperm and to the higher incidence of hypogonadism and cryptorhism.[27,28,30]

Since estrogens are related to the genesis of other types of cancer, over 2200 mothers of the boys who developed testicular cancer, were studied in Denmark. The study revealed no higher incidence of breast cancer, of endometric or of ovarian cancer in them, so that these women did not represent a higher risk group of oestrogen related cancers.[31] Indirectly, this could mean that estrogens may not play a role as important as was thought in the genesis of testicular cancer. Such a conclusion would be premature, however, since countries with a higher incidence of breast cancer (which is likely estrogen related cancer) also report a higher incidence of testicular cancer, and it would suggest some relation between the two.

Social factors

The incidence of testicular cancer is higher in men of higher social status.[1] We detected a relatively high percentage (40.2%) of patients with college or university education.[10] A better social status may be related to more frequent diaper changes and consequently higher temperature in the genital area during the first months of life. The influence of temperature on the incidence of testicular cancer has not been studied enough.

The impact of trauma

The impact of trauma on the development of testicular cancer has not been cleared. Most probably, trauma does not raise the incidence of testicular cancer. The fact that a larger number of patients mention trauma in their

anamnesis can be ascribed to the acute attention paid to a blows received in the genital area. Patients may discover at that point tissue hardening or swellings.[10,32]

Despite the above, certain sports (cycling and horse riding) could cause injuries related to the development of testicular cancer.[33] It is possible that any type of injury could act as an additional conducive factor to the already existing cause. Swerdlow reports an increased incidence of testicular cancer when biopsy was performed in addition to orchidopexy.[34]

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Lymphotropic staining of the sentinel lymph nodes in breast cancer - with what, when, how?

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The aim of the study is to define how to choose appropriate dye for marking sentinel lymph nodes in breast cancer. Pre- or intra operatively, dyes, such as Methylen Blue, Drimaren Brilliant Blue, Patent Blue V were applied around the tumor in 135 female patients. To enhance lymphotropism, Gelatin, Alvezin, Haemodex or HAES solutions were used as dye carriers in 92 patients. The volume applied varied from 1.0 to 3.0 ml, as in 25% of the cases Hylase was previously applied to increase absorption. The study also included 29 patients in whom a preoperative chemotherapy with Mitoxantrone was carried out, the cytostatic blue color being used for identification of the first filtrating lymph vessels and nodes. Most frequently, visualization was achieved with Mitoxantrone (80% of cases), Patent Blue V (76%), and the combination of Drimaren with Haemodex (57%).

Key words: breast neoplasms; lymph nodes - anatomy and histology; staining methods

Introduction

The small size of the axillary lymph nodes, their colourlessness, location in the fatty tissue as well as some anatomic-topographic features have necessitated the search for staining methods which would ensure a more precise indentification.[1]

Until recently, perioperatively use of different dyes, such as Sky Blue, Pontamine Sky Blue etc, injected intramammarily, or the direct colour lymphography with either Lymphotrast or Chromolymphotrast, were used to visualize lymph nodes in order to facilitate their radical treatment.[1,2]

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Adopting the hypothesis of the succession of axillary metastases from level one to three, and in search for less invasive methods for early breast staging, some reports for identification of the first lymph nodes draining the primary tumor (so called sentinel lymph nodes) by applying variouns dyes have appeared during the last years.

According to A. Giuliano[3,4] and A. Barth[5] after a peritumor application of Isosulfan Blue or Patent Blue V, it is possible to follow up the lymph vessels and the first lymph node (nodes), located on the way of the lymph drainage and after staining them to identify and histologically examine them. Selective biopsy from this "first station"of metastasis and the results obtained from this "strategic side" - negative or positive nodes,

determine the necessity for axillary lymph node dissection.

The wide variety of dyes used, the perspective by carriers with molecular weight over 10 000 to improve the selective transport (lymphotropism),[6,7] as well as the timing of application: from 18 h before surgery to intraoperative use, grounded our decision regarding the possibilities of lymphotropic marking of sentinel lymph nodes in breast cancer.

Materials and methods

The study included 135 female patients with breast cancer (stages I and II) treated at the University Oncology Center-Pleven during the period January 1995 - April 1997, where pre- or intraoperatively, different lymphotropically dyes were applied around the tumor independently or by carriers (Table 1).

Table 1. Dyes used in patients with breast cancer

Dyes	Producer	Number of cases		
		independently	with carrier	total
Drimaren Brilliant Blue	Fluka - Cat N 44582 1% solution in PBS	10	39	49
Methylen Blau VITIS	Neopharma amp. 1% 5 ml	8	31	39
Patent Blue V	BYK Gulden amp. 2,5% 2ml	25	22	47
Total		43	92	135

To enhance dye lymphotropism, several carriers were applied together with the dye in ratio 1:1 or 1:2 (Table 2).

The quantity of dye applied around the tumor independently or by carriers varied from 1.0 to 3.0 ml, and immediately after application, the site was gently massaged for several minutes.

Based on studies proving the role of Hyaluronidase for increasing the macromolecule transportation from interstitial space to lymph vessels,[1,7] 39 patients were treated with Hylase (Hylase Dessau, Germed). A total dose of 50-100 U was injected around the tumor and several minutes later via a needle in the same site the dye with a carrier was introduced.

The study also included 29 females with breast cancer (stages I and II) in whom locoregional perioperative chemotherapy with Mitoxantrone (Novantrone, Wyeth-Lederle) had been carried out. The cytostatic was used immediately before the introduction of anaesthesia. Two sites around the tumor in the lateral pole of the carcinoma (lateral localization - 18 cases) and around the four poles (central and medial localisation - 11 cases) were injected with 0,5 ml (1mg) Mitoxantrone.

All 164 patients who had a lymphotropic staining done were operated on. A modified radical mastectomy was done in 132 (T>1 cm) patients, and quadrantectomy with axillar lymph dissection - in the rest 32 (T≤1 cm) patients. The intraoperatively found sentinel lymph nodes were biopsied, submitted to histological examination and compared to the results obtained on dissection.

Results

During surgery an intensive staining with a diameter from 2 to 4,5 cm was found in the sites of application and in about a half of the cases dye filled lymphatic tracts were identified at a distance of 2 - 4 cm from the primary site of injection.

Sentinel nodes colored by Mitoxantrone after perioperative locoregional chemothera

Table 2. Carriers applied for enhancement of the selective dye transportation

Carrier	Producer	Characteristics	Number of cases
Haemodex 40	Troyapharm banks x 500 ml	Solution of dextran, mean molecular weight 40,000 in isotonic solution of NaCl	30
Alvezin 40	Berlin-Chemie AG, banks x 1000 ml	L-aminoacid infusion solution with theoretical osmolarity 801,8 mosmol/l.	28
HAES-Steril 10 %	Fresenius AG banks x 1000 ml	10% solution of modified starch with mean molecular weight 200,000 in isotonic NaCl solution	20
Gelatin	Sigma cat N 62625	0,1% PBS solution with molecular weight approximately 80,000	14

Table 3. Distribution of the sentinel lymph nodes according to the dyes and carriers used.

Dyes	Independently	Carriers				Marked sentinel nodes		Number of cases with uninstained lymph nodes
		Haemodex	Alvezin	HAES	Gelatin	Number of cases	Number of lymph nodes	
Drimaren	10					3	5	7
Briliant		14				8 (6)	10	6 (2)
Blue			10			5 (3)	7	5 (2)
n=49				9		3 (2)	4	6 (2)
					6	—	—	6 (2)
Methylen	8					2	3	6
Blue		9				3 (1)	4	6 (2)
n=39			11			3 (2)	5	8 (1)
				6		2 (1)	3	4 (1)
					5	2	3	3 (2)
Patent	25					19	28	6
Blue		7				5 (3)	7	2
n=47			7			5 (3)	8	2 (1)
				5		3 (2)	4	2 (1)
					3	1	1	2
Mitoxan trone n=29	29		—			23	40	6
Total n=164	72	30	28	20	14	87 (23)	132	77 (16)

Note: Hylase was used to 39 cases treated with carrier (they are marked in brackets) - in 23 of them stained sentinel lymph nodes were observed and in 16 - unstained.

Table 4. Distribution of the cases with lymphotropically used carrier according to the time of application.

Lymphotropic application of:	Time of application				Total number of marked cased	Number of uninstained nodes
	intraoperatively	before surgery				
		1-3h	12h	20h and 1-3h		
stained independently n=43	10(5)	19(12)	5 (1)	9(6)	24	19
stained with carrier n=92	17 (4)	40 (18)	16(4)	19(14)	40	52
Mitoxantrone n=29	4 (1)	12(10)	4(3)	9(9)	23	6
Total n=164	31 (10)	71(40)	25(8)	37(29)	87	77

Note: Patients with marked lymph nodes are enclosed in bracket.

py were the most frequently observed (in 23 out of 29 cases - 80%), followed by the lymphotropic application of Patent Blue V (in 19 out of 25 cases - 76%) and Drimaren with Haemodex (in 5 out of 9 cases - 55.5 %).

The distribution of the sentinel lymph nodes according to the dye used is presented on Table 3.

Patients are divided by the time of dye application. Data are seem in Table 4.

An insignificant local erythema, without subjective complaint was observed in 5 patients: in 3 with used Mitoxantrone and in 2 with Metylen Blau and Alvezin.

Discussion

Our results (identification of sentinel lymph nodes in 87 out of 164 cases with breast cancer) are close to those reported by A. Giuliano[3] - 114 out of 259 cases after application of Patent Blue V or Lymphazurin. In 77 of the cases with an identified sentinel lymph node, the latter is located on axillary level I; in seven cases with 2 stained lymph nodes, one is on level I and the other on level II; in three cases the sentinel nodes are on level II.

The highest rate of sentinel lymph nodes detection was achieved when Mitoxantrone was used. Our clinical observation on Mitoxantrone supports the reports of its comparatively good local tolerance,[8] and its good absoption from the regional lymph nodes draining the injection site.[9]

The results obtained with Patent Blue V supported its good lymphotropism, which renderd it an universal dye in the initial stage of direct contrast lymphography, where it was used for the detection and cannulation of lymphatics.

Additional investigations on a greater number of females are required to confirm the encouraging initial results obtained by the use of Drimaren Brilliant Blue with carrier Haemodex or Alvezin. In the available literature there are no reports on the Drimaren application for sentinel lymph node mapping.

In peritumor injection of Methylen Blau (alone or with carrier) an intensive local staining of the adjacent parenchyma was observed, but a comparatively poor penetration into the draining lymph nodes and vessels was found. These observations are consistent with the experimental study of J. Wong.[10]

Insignificant erythema after application, described as a side effect is rare and coincides with the reports of other authors.[10]

Conclusions

Lymphotropic staining:

With what?

Our clinical observations point out that Mitoxantrone and Patent Blue V are most appropriate for the identification of the sentinel lymph nodes in breast cancer. An advantage of the chemotherapeutic represents the possibility for locoregional control. improvement.

When ?

The most appropriate time for application is: either twofold - 20 h and 1 -3 h before surgery, or single time 1-3 h before surgery. Especially for Patent Blue V only an intraoperative application is relevant.

How?

An injection of Hylase before the application of dye with carrier enhances their resorption from a peritumor depot and improves the sentinel lymph node mapping.

The study continues according to the preliminarily defined program.

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Carcinoma of the thyroid: Postoperative radiotherapy

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We report a retrospective analysis of patients with thyroid cancer who received postoperative external radiotherapy (RT) and high-dose radioiodine. Between 1982 and 1993, 69 patients with thyroid cancers of four histologic types (follicular, n=17; papillary, n=39; medullar, n=7; anaplastic, n=6) were treated by surgery, ablative radioiodine therapy (only differentiated tumours) and radiotherapy. Indications for adjuvant radiotherapy were extraglandular tumour infiltration (stage pT4) and/or incomplete surgical resection and/or extensive lymph node involvement or difficult surgical excision for repeated local recurrences. Radiotherapy was delivered by parallel opposed fields (50 Gy, 2 Gy) in a Amini-mantle-technique"; this was followed by an electron-boost (10 to 14 Gy, 2 Gy) using a midline wax bolus. The adjusted 5-year survival rate was 82.6%, the 5-year disease-free survival rate 92.1% (mean observation time 56 months, range 4 to 146 months). Statistically significant relationship was observed between adjusted survival rate and parameters like histology (p<0.00001), cervical lymph node involvement (p<0.002), metastases at presentation (p<0.001) and age (p=0.03). No severe radiation induced late complications -specifically of the spinal cord or the trachea - were recorded. Postoperative high dose radiotherapy (60 to 64 Gy), in case of differentiated thyroid tumours in combination with high dose radioiodine therapy, seems to be an effective tool to sterilise microscopic or macroscopic residual disease and can safely be delivered using modern radiation treatment techniques.

Key words: thyroid neoplasms - surgery; radiotherapy, adjuvant

Introduction

Thyroid cancer, which accounts for less than 1% of all cancer deaths[1] is described more frequently in females than in males (ratio about 2-3:1). There are four different histologic types with distinct different clinical behaviour. Papillary tumours are generally observed in young and middle-aged adults. Metastases are generally found in the lymph nodes of the neck ipsilateral to the primary lesion. Follicular thyroid carcinomas occur in a slightly older population and usually metastasise haematogeniously to distant sites with lymph node metastases being uncommon. Both so called differentiated thyroid cancers

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arise from the thyroid follicular cells while medullary carcinoma of the thyroid derives from the ĄC" - or parafollicular cells of the thyroid gland. In the medullary type tumour cells spread over blood and lymphatic routes producing metastases in the cervical lymph nodes, the lung, liver and bones. The aggressive anaplastic thyroid carcinomas occur primarily in patients older than 50 years of age, and are characterized by a rapid local growth. This tumour type mainly presents lymph node metastases and lung metastases in an early stage of disease.

The surgical resection is considered the method of choice for initial treatment of differentiated and medullary thyroid cancer as well as anaplastic tumours if the radical resection or even tumour debulking are technically feasible. The ablation of residual remnants with radioiodine usually follows in patients with differentiated thyroid cancer since that will permit subsequent whole body scintigraphy to exclude the presence of residual or metastatic disease. The value of postoperative radiotherapy (RT) is still discussed controversially. Some reports described a reduction of local recurrence, particularly after the incomplete surgery[2-8] or even a better survival rate following postoperative radiotherapy for all histologic types of thyroid cancer.[9,10] In contrary, other authors found therapeutic improvement only in specific histologic subgroups.[11,12] Other studies have denied any effect on survival[13-15] or even revealed worse results in patients treated with radiotherapy postsurgically.[16]

We present the results of a retrospective analysis of 69 patients with thyroid cancer who received postoperative radiotherapy using the wax bolus technique.

Patients and methods

Patients

Between 1982 to 1993, 69 patients (50 women, 19 men; mean age 53,5 years; range 19 to 86 years) with thyroid cancers were included in our study. Seventeen patients (13 women, 4 men) were younger than 40 years of age and 52 patients (37 women, 15 men) were older than 40 years at the time of diagnosis.

Criteria for adjuvant postoperative radiotherapy were defined either as macroscopically or microscopically incomplete surgical excision and/or tumour invasion in neighbouring structures (pT4) and/or extensive lymph node involvement or upper mediastinal involvement. Six patients (8.6%) presented with distant metastases at the time of referral. No patient had a history of prior external beam irradiation of the neck.

All patients underwent surgery; total thyroidectomy was performed in 25 patients (36%), subtotal thyroidectomy in 26 patients (37.5%), unilateral lobectomy in 9 patients (13.0%) and in the remaining 9 patients (13%) only a palliative tumour excision was possible. Six patients (8.6%) underwent the surgical excision of locoregional recurrence. In case of clinically/sonographically enlarged cervical lymph nodes a modified unilateral neck dissection was performed in 23 patients (33.1%) and a bilateral neck dissection in 3 patients (4.3%). In 17 patients (24.5%) only singular enlarged cervical lymph nodes suspected for tumour infiltration were excised (Ąberry picking"). In the remaining 26 patients (37.5%) no lymph nodes were surgically removed.

The tumour-node-metastasis classification of malignant tumours of the International Union Against Cancer (UICC) was used for the postoperative pathological staging as summarised in Table 1. All tumour stages were reclassified according to UICC 1992.[17] In 13 patients (18.7%) the excision of the

Table 1. Postoperative TNM (UICC) classification

T	< 40 years of age			> 40 years of age		
	N			N		
	0	1	X*	0	1	X
1	0	2	0	1	2	0
2	1	3	1	1	3	3 (1)**
3	0	1	0	2	4	4 (1)
4	3	5	1	5	8(2)	14(1)

NX* : No lymph nodes surgically removed
(1)**: Patients with distant metastases at diagnosis

tumour turned out to be macroscopically incomplete, in 23 (33.1%) patients microscopically. A histopathological examination of the specimens revealed 39 papillary carcinomas, 17 follicular carcinomas, 7 medullary carcinomas, and 6 anaplastic carcinomas. Papillary cancers included both, purely papillary and mixed papillary-follicular tumours.

After the surgery, the ablative radioiodine treatment was performed in 56 patients (80.6%) using a mean dose of 2,8 GBq. Six patients were treated with a second ablative radioiodine therapy for thyroid remnants. Six more patients received radioiodine treatment for loco-regional tumour recurrence and/or distant metastases additionally.

During the follow-up period 8 patients (11.5%) were treated with sequential chemotherapy regimes. All patients underwent T 4 suppression therapy.

External radiotherapy

The adjuvant radiotherapy was delivered with Co^{60} teletherapy in 88% and photon beams (8MV) in 12% of patients with a continuous course of 46 to 50 Gy, 2 Gy/d, 5 d/wk. Treatment fields encompassed all lymph nodes from the base of the skull to the upper part of the mediastinum with a boost to the actual or potential area of residual disease. The radiation treatment was carried out with opposed anterior/posterior fields in a "mini-mantle"- technique with shielding to the floor of the mouth and subapical portions of the lung. The spinal cord was blocked out from the posterior field after 42 Gy. Routinely, the treatment was based on CT planning. The dose was described to the isodose encompassing the clinical target volume (minimum 85%).

This was followed by an anterior electron boost (15 or 20 MeV) with given doses up to 60 to 64 Gy, 2 Gy/d. For each patient an individually wax bolus was manufactured to yield a maximum dose at the target volume and a minimum dose at the spinal cord. During the electron boost treatment this bolus was applied to the patient's neck to guarantee an optimal dose distribution (Figure 1, 2). In patients with tracheostomy (n=4) the wax

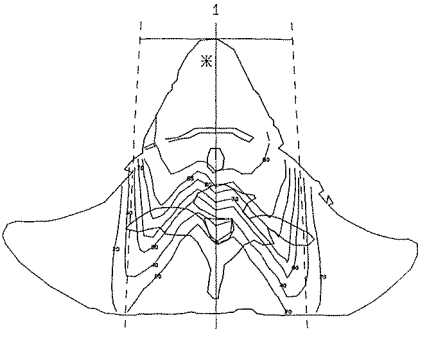

Figure 1. Dose distribution in the transversal plane using 15 MeV electron beams.

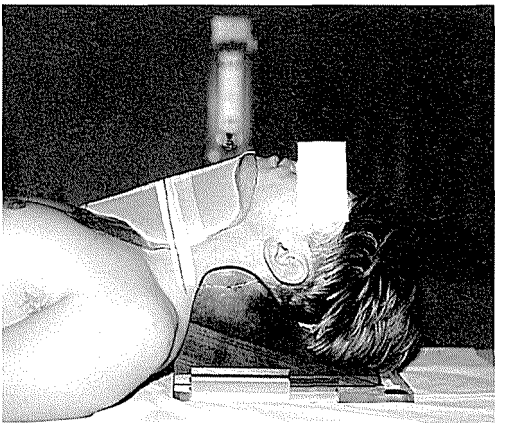

Figure 2. Positioning of the wax bolus during electron boost treatment.

bolus was not applied. In such cases the boost was given through two lateral shaped portals.

Follow up

The follow-up investigations included monitoring of thyroid hormone and thyreoglobulin/calcitonin levels, sonography of the neck, radionuclide (I^{131}) imaging studies and chest radiographs and were performed at 3- or 6-month intervals during the first 5 years and thereafter annually. The computed tomography (CT) of the neck and the chest were not performed routinely. The recurrent disease or metastases were confirmed by biopsy, if clinically feasible. Otherwise the radiological (I^{131}-whole body scan, x-ray films, CT scans) evidence of recurrence and metastases was accepted.

Data analysis

The survival curves were calculated using the Kaplan-Meier product limit method. Events for relapse-free survival were locoregional recurrence or metastasis. Survival and time to relapse were calculated from the end of the adjuvant RT. For the comparison of categorical variables, the chi square test was used. A two-sided probability level of <0.05 was considered significant. A statistical analysis was done using a software program (SPSS; Spectra Publishing; Sunnyvale, Calif.).

Results

Survival rate

The adjusted 5-year survival rate (mean observation time 56 months; range 4 to 146 months) of all patients was 82.6%. The histologic subtype was a strong determinant for survival ($p<0.00001$). Papillary carcinoma patients had a 5-year adjusted survival rate of 97,3%, follicular carcinoma patients of 76,6%, medullar cancer patients of 83.8% and anaplastic carcinoma patients of 16.7%. 11/69 patients (15.8%) died of the thyroid malignancy. All of these patients were more than 40 years of age and had tumour stage pT4 and/or M1 at the time of diagnosis. 5/69 patients (7.2%) died due to the unrelated disease without any evidence of tumour after 12, 22, 27, 50, and 74 months, respectively.

Statistically significant relationships were also found between adjusted survival rate and parameters like age ($p=0.03$), cervical lymph node involvement ($p<0.002$) and distant metastasis at the time of diagnosis ($p<0.001$).

Local recurrence rate

The 5-year disease-free survival rate of all patients was 92.1%. The local control rate was 100% for patients younger than 40 years of age including the salvage treatment. In patients older than 40 years locoregional recurrence within the irradiated field was observed in 6/52 patients (11.5%). Details are given in Table 2.

Only distant metastases at diagnosis were found to have an influence on the risk of locoregional recurrence ($p=0.04$, significant).

Table 2: Rate of recurrence and follow up

TNM	Hist.	Site	Time	Treatm. (months)	Outcome	(months)
f [34]	T4 N1	med	LR	4	"S, RT"	
				22	C	95 [NED]
m[46]	T4 N1	foll	lung	7		
			liver; skin	28; 46		56 †
f [68]	T3 NX*	foll	LR	6; 11	RI	
			lung	15;22;34	RI	
			bone	44	RI	44 PR
f [65]	T3 NX	foll	lung	62; 77	RI	79 [PR]
m[57]	T4 NX	foll	LR	111;117	"S, RI; RI"	125 [NED]
f [60]	T4 NX*	pap	lung	42	"S, RI"	
			lung	49	C	65 [PR]
f [77]	T4 NX	pap	lung	110		130 †
m[46]	T1 N0	med	LR	3; 13	"S, RT;C"	93 [SD]
m[53]	T4 N1	ana	liver	23	C	34 †
f [59]	T4 NX	ana	lung	1		9 †
f [66]	T4 NX	ana	lung	3		17 †
f [7]6	T4 NX	ana	lung	31		57 †
f [78]	T4 Nx	ana	lung	3		4 †
m[56]	T2 NX* M1	foll	LR	20	"RI, C"	
	(lung)		bone	31		39 †
f [80]	T4 N1 M1	foll	lung [PD]	2	C	17 †
	(lung)					
f [49]	T4 N1 M1	pap	lung [NC]	3	RI	51 [NED]
	(lung)					
f [67]	T3 NX M1	pap	liver	20		28 †
	(bone)		lung	21		
f [50]	T4 N0* M1	med	bone [PD]	12	"RT,C"	
	(bone)		lung; LR	17; 25		37 †
f [74]	T4 NX M1	med	lung [PD]	5	C	74 †
	(lung)					

"f [34]: Female, 34 years of age"
"m[46]: Male, 46 years of age"
med: Medullary thyroid cancer
pap: Papillary thyroid cancer
foll: Follicular thyrpoid cancer
ana: Anaplastic thyroid cancer
RT: Radiotherapy
RI: Radioiodine
S: Surgery
C: Chemotherapy

*Surgery for local recurrence before referral
NED: No evidence of disease
NC: No change
LR: Local recurrence during followup
SD: Stable disease (localregional)
PR: Partial remission
PD: Progressive disease
†: Dead of disease

Distant metastases

In patients younger than 40 years of age no distant metastases occurred, whereas in patients older than 40 years of age distant metastases were observed in 10/52 patients (19.2%). Another three patients with distant failure at the time of diagnosis developed distant metastases on other anatomic sites during the follow-up period.

The rate of recurrences (taking into account age at diagnosis, histological type, site and treatment of recurrence, and follow-up for patients without and with distant metastases at referral) are displayed in Table 2.

Side effects of external radiotherapy

Acute side effects like the radiation induced laryngeo-pharyngitis and tracheitis as well as dermatitis occurred in all patients, particularly in the later phase of the external radiotherapy. Glottis oedema was seen in 3 patients (4.3%). Two patients (2.9%) experienced mild Lhermitte Syndrom but they recovered completely. Fifteen months after the radiotherapy of a gross residual anaplastic thyroid carcinoma an esophagotracheal fistula occurred in one patient (1,4%) and required percutaneous gastrotomia. Serious late irradiation induced complications of the spinal cord or the trachea were not observed.

Discussion

For many years the postoperative radiotherapy has been frequently used to prevent local failure for example in patients with breast cancer, rectal cancer or soft tissue sarcomas. The rationale of postoperative RT in patients with thyroid cancer has also the goal to prevent a local recurrence and to increase a long term survival rate. Because of the rarity of this disease and its long natural history, the evaluation of the value of postoperative RT in thyroid cancer relies mostly on retrospective studies.

The role of the adjuvant RT in papillary and follicular, so called *differentiated thyroid carcinomas,* has remained an issue of controversial discussion. Tubiana et al.[8] reported a highly significant difference in the total number of local recurrences between patients treated by surgery alone or by a combination of surgery and radiotherapy. The difference could be demonstrated in patients after the complete surgical treatment (21% vs. 14%) as well as in patients after the incomplete surgical excision (32% vs. 15%). Furthermore, the authors observed a statistically significant difference in the number of recurrences between patients who received a high-energy beam irradiation with an adequate doses of > 50 Gy compared to those with a conventional x-ray treatment with lower doses. Other previous reports describe better local control rates after a high-dose radiotherapy resulting in the improved survival rates.[9,10] the better survival rates only in patients with papillary thyroid cancer,[11,12] or the improved local control rates which did not translate into a survival benefit.[2] In 1990 Benker et al.[18] observed no beneficial effect of adjuvant radiotherapy on the survival in patients with T1-T4 tumours. The same data were reanalysed in 1996 by Farahati et al.[12] focusing only on patients with T4 tumours and an improved recurrence-free survival in patients older than 40 years with papillary histology and lymph node involvement was found.

Some authors believe that the ablative radioiodine therapy alone without a postoperative radiotherapy is sufficient for the tumour control particularly in cases of the differentiated thyroid cancer with micrometastases or microscopical residual tumour.[13,14] However, using a dosimetry simulation by Monte Carlo techniques Sauter-Biehl et al.[19] found that in tumours of r = 0.5 mm or less the percentage of a total dose deposited inside the tumour rapidly decreases and extensive dose inhomogeneities

appear. The authors pointed out that in small tumours like micrometastases in lymph nodes or in the tumour bed tumoricidal doses would not necessarily be achievable by radioiodine alone and the additional postoperative RT would be necessary to achieve a tumour control.

Contradictory results have also been observed for the effectiveness of the adjuvant RT in medullar carcinoma. Saamann et al.[16] described a lower survival rate in patients with a postoperative radiotherapy compared to those without this treatment. In this study, however, fatal recurrences in the irradiated patients occurred outside the radiation portals. In contrast to these results, other authors[3-6] found the adjuvant external irradiation with sufficient doses beneficial in preventing local recurrences in high risk patients with extraglandular invasion (pT4), particularly with lymph node metastases or after the incomplete surgery.

The local control can be achieved in the anaplastic carcinoma using radiotherapy, but the survival is usually low since many patients die of the disseminated disease. Improved results have been observed after the multimodale treatment with a combination of surgery, chemotherapy and adjuvant radiotherapy,[20-23] particularly with the hyperfractionation.[23,24] In patients with anaplastic carcinomas who lived longer than 12 months (Along survivors") Aldinger et al.[20] found histologically small areas of spindle or giant cell carcinomas while the rest of the tumour mostly consisted of well differentiated tumour cells. This observation was confirmed in our own small series of six patients in three "long term survivors" with the survival of 17, 57 and 74 months, respectively.

In the current analysis patients with thyroid cancer of all four histologic types were evaluated. To obtain a satisfactory rate of the locoregional control high radiation doses are required.[4-6,8,10,12,25] therefore a total dose of 60 to 64 Gy was chosen in order to deliver a dosage which is able to sterilise residual tumour cells - either in the tumour bed or in the lymph nodes. In our patients we observed a low local recurrence rate of 11.5% in patients older than 40 years of age which is comparable to data from the literature.[4,5,8,12,25] Various techniques have been advised to deliver a high dose to this region.[26-28] After computer assisted treatment planning parallel opposed $Cobalt^{60}$-or megavoltage photons fields in combination with electrons were used to deliver a sufficient dose to the target volume. Using an individual formed wax bolus during the anterior electron boost field made it possible to shape the electron beam isodose curves around the vertebral body to irradiate the tumour bed with a sufficient dose and to protect the spinal cord and the trachea as much as possible. Using this technique, no serious late irradiation induced complications were encountered.

Conclusion

We conclude that there is an estimable role for the adjuvant external radiotherapy after the ablative radioiodine treatment in the management of patients with differentiated thyroid cancer in stage T4 N0-2 as well as in patients with microscopic or macroscopic residual disease. In stage pT1-3 and lymph node involvement as well as in the medullary thyroid cancer treatment a decision has to be made individually considering risk factors e.g. the age of the patient, the completeness of a resection and the extent of a lymph node involvement. The combination modality treatment including surgery, chemotherapy and (hyperfractionated) external radiotherapy remains the treatment of choice in patients with anaplastic thyroid cancer to obtain a better local control and to diminish distant metastases.

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Pectoralis major flaps for reconstruction of the head and neck defects

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Between 1991 and 1995, the pectoralis major myocutaneous flap used for the reconstruction of defects in the head and neck region in 26 and the pectoralis major osteomyocutaneous flap in one patient were treated at Ankara Oncology Hospital. The mandible was resected in 13 patients who suffered from the destruction of the bone. Among these patients segmental mandibulectomy was performed in nine patients, marginal mandibulectomy in seven patients and hemimandibulectomy in one patient. Peroperative mortality was 3.7%. A recurrence at flap region was seen in six patients and a mandibular deformity occurred in six cases. In conclusion the pectoralis major osteomyocutaneous flap is a reliable flap that can be used for the immediate reconstruction in the head and neck region.

Key words: head and neck neoplasms surgery; surgical flaps; mandible-surgery; pectoralis muscles

Introduction

The reconstruction of large soft tissue defects after the ablative cancer surgery, especially in the head and neck region, is a significant problem which turns out to be even more serious when the mandible is resected. The goals of the reconstruction in the head and neck region should be directed: (1) to achieve a healed wound and not to delay the adjuvant therapy; (2) to maximize the tongue function and consequently the deglutition and the speech; and (3) to restore cosmesis.[1]

In the retrospective chart review, the authors present their experience in the treatment of 27 patients in whom large defects after the ablative cancer surgery in the head and neck region were primarily reconstructed with pectoralis major flaps.

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Material and methods

Medical records of all patients who underwent a PMMC or PMOMC flap reconstruction for soft tissue defects after the ablative cancer surgery in the head and neck region between 1991-1995 at Ankara Oncology Hospital were reviewed. The data were analyzed with respect to the demography, the type of operation, complications, and results.

The operative technique for PMMC or PMOMC flap was similar to those for the standard technique that had been reported previously.[2,3]

Results

Between 1991 and 1995, 26 PMMC flaps and 1 PMOMC flap were used for the reconstruction of post-ablative defects in the head and neck region. Among the treated patients there were 20 males and 7 females. The mean age was 53 (range 35-70). The distribution of the primary tumor sites of the 27 patients is presented in Table 1. The histopathologic types of the tumors were squamous cell carcinoma in 21 patients, malignant melanoma in three patients, ameloblastoma in one patient, thyroid papiller carcinoma in one patient and malignant epithelial tumor in one patient. Eight patients in the present series received a previous radiotherapy.

Table 1. Distribution according to the type of primary tumors

Lower lip	12
Metastatic mass in neck	4
Carcinoma of skin	4
Carcinoma of larynx	2
Carcinoma of tonsil	1
Ameloblastoma	1
Carcinoma of tongue	1
Carcinoma of floor of mouth	1
Carcinoma of ear and temporal bone	1

The mandible was resected in 13 patients who suffered from the destruction of the bone. Among them nine patients suffered from the segmental mandibulectomy, three from marginal mandibulectomy and one from the hemimandibulectomy. For the mandibular Kirschner wires were used in seven cases, rib in one and titanium replacement plates in two cases. The fracture of the mandible occurred in one of the patients who suffered from the marginal mandibulectomy.

The treatment modalities are shown in Table 2. In two cases an early flap necrosis occurred. One of these cases was lost with respiratory distress and the other one underwent trapezius myocutaneous flap reconstruction after this complication. The average follow up period was three years.

The peroperative mortality was 3.7% (1/27 patients). In the follow up period, mortalities occurred one month after the operation in one patient and 6 months after the operation in two patients due to the neutropenic sepsis.

A recurrence at the flap region was seen in six of 27 patients. A mandibular deformity occurred in six patients who had undergone the mandibular reconstruction with a stainless steel wire.

Discussion

The reconstruction of large defects after the resection of tumors in the head and neck

Table 2. Treatment Modalities in patients

RND + Wide excision + PMMC	8
RND + Mandibulectomy + PMMC	8
RND + Mastoidectomy + PMMC	1
RND + Mandibulectomy + par. glossectomy + PMOMC	1
RND + Mandibulectomy + excision of floor of mouth	1
RND + Laryngectomy + Oesephagectomy + PMMC	2
Wide excision + Mandibulectomy + PMMC	2
Wide excision + PMMC	3
Composite resection (Jaw-Neck) + PMMC	1

RND: radical neck dissection , PMMC: pectoralis major myocutaneous flap , PMMOC: pectoralis major osteomyocutaneous flap

area has been facilitated by the development of myocutaneous flaps.[2] The use of myocutaneous flaps, which provide both the muscle bulk and the skin coverage, represents a significant advancement in the reconstructive surgery.[3] Traditionally, the reconstruction in the head and neck region after extensive resections for malignancy had been accomplished by the use of forehead flaps, deltopectoral flaps, and shoulder flaps.[4]

The forehead flap described by McGregor has been used previously, but the donor site is cosmetically unappealing, and the flap insertion can be difficult because of the pedicle bulk.[1,5] The deltopectoral flap described by Bakamjian has also been used, but the precarious axial blood flow and the necessity of multiple procedures limit its availability.[1,4,5] The trapezius myocutaneous flap often requires two stages and also leaves a significant donor defect, which needs a skin graft.[5] Microsurgery was performed widely in the mid 1970s, however, the success of free flaps was rapidly overrun by the implementation of musculocutaneous flaps.[1,6] In the musculocutaneous flaps the dissection is easy and the surgeon does not need microsurgical experience.[1,2]

Although Hueston and McConchie described the use of the pectoralis major myocutaneous flap in the reconstructive surgery in 1968, its use in the head and neck region has not been reported until 1979.[3,4,5,7,8] The anatomical basis and operative techniques of the PMMC and PM-OMC flaps have been well described in the literature.[7,8,9] The pectoralis major muscle has been shown to be useful as a muscle and myocutaneous flap unit for defects of the head and neck.[10] Its dual blood supply from the thoracoacromial artery and from the perforating intercostal branches of the internal thoracic artery provides a considerable versatility in orientation and configuration.[7,10,11] The thoracoacromial vessels have a consistent origin in the axillary vessels and are rarely included in the radiation field in head and neck malignancies.[8] This flap procedure has also been performed without any technical problems in eight cases who had radiotherapy to the neck, similar to those previously reported.[3] The blood supplies of the skin paddle of the flap and the rib in PMMC and PM-OMC flaps are provided by the muscle perforators and the periosteal blood vessels, retrospectively.[8,12] "Andy Gump" deformity due to a progressive resorption of the rib was reported as a long term complication of the patients with PMOMC flap.[13] We have not noticed this complication in our series. This may be due to the short duration of the follow up period of only one patient with PMOMC flap. A mandibular deformity occurred in many of our patients in whom their mandibles were reconstructed with a steel-wire, so in our opinion it will be more appropriate to use a mandible prosthesis in the mandibular reconstruction.

A series of patients with cancer in the head and neck region have undergone the immediate reconstruction with the PMMC flap.[4,14,15] But it has been reported that PMMC flap is not an ideal reconstruction for intraoral tumors because of several complications such as a stricture and an orocutaneous fistula formation.[3,15] However, patients suffering form the advanced stage cancers require a wide resection with the reconstruction which may be achieved with PMMC flap. Our patients suffering from the oral cavity cancers underwent no such complications. This may be related to the attention that has been paid to the tension of the suture line.

The PMMC flap is a method that has several advantages:[2] 1-It is an axial flap, with an excellent blood supply to the muscle and overlying skin. 2-It can be used to transport a large amount of muscles for bulk, and an attached rib for bone graft. 3-The muscle portion not only covers the carotid artery but also provides bulk to fill the hollow and restore the contour after a neck dissection.

4-The flap has enough length to provide a coverage to distant sites such as the fronto-orbital and temporo-parietal areas. 5-The donor site can be closed by expanding the chest skin locally. Furthermore, this flap may be performed with minor morbidity and without any sever long term complications. In our series we have seen two flap necrosis due to ischemia in patients who previously underwent the operation.

In conclusion, the PMMC flap is a reliable, versatile flap that can be used for the immediate reconstruction of a variety of defects at different locations in the head and neck region. Our experience suggests its use in restoration of soft tissue defects after the intraoral cancer ablation.

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Report on the Workshop on Strategy of Investment in Radiological Equipment for the Central and Eastern European countries held in Baden bei Wien 27-28 September 1997

European Association of Radiology (EAR)

In 1995 the European Commission suggested to EAR that it should arrange a meeting to discuss the investment in radiological equipment in Central and Eastern European Countries, (CEEC), and to compare it with the strategies for such investment in Western European countries.

EAR welcomed the suggestion, and the Workshop took place on the above dates. The arrangements were made by Prof. M. Bléry (France), as Secretary-General of EAR, and Prof. A.L. Baert (Belgium), as Past-President of EAR with Prof. R. Rienmüller (Austria) undertaking the detailed local arrangements, with the support of the ECR office. Invitations were sent to members of the EAR Bureau, representatives from DGI and DG XIII of the EC, senior radiologists from Germany and Italy (as indicative of practice in Western Europe), senior radiologists from several CEEC, and representatives of the radiological industry.

Prof. H. Ringertz (Sweden), President of EAR, welcomed those present and invited them to discuss aspects of national parameters which may influence investment in medicine, especially in radiology.

Dr. H. Bourgade (DGI of EC), explained the conditions which control the PHARE (Poland, Hungary Assistance for the Restructuring of the Economy) programme, and how they had been changed since the start in 1990, from maintenance of Basic Services through material support of the restructuring and reforming of the Health Sector to demand-driven approaches from countries involved within the frame of he PHARE Health Strategy, including a continuing increase in the number of countries involved, now amounting to 13. She underlined the need for realistic plans involving education and professional handling of the equipment installed in the respective countries. She noted that the EU could not work through an organisation like EAR, but only through national governments, which can be influenced by National Societies, and that it took into account internal efforts being made by the countries themselves, their requests and any progress made towards co-ordination with other possible partners. She strongly warned against a too naive "gift" mentality which she had recognised regarding other parts of the world.

Dr. Beolchi, (DG XIII), explained the development of the Healthcare Telematics programme within EU, from "industrial quality" towards "clinical quality" by the year 2000.

Mr. Holsten explained the EC-promoted exercise TACIS, (Technical Assistance for he Commonwealth of Independent States), to evaluate the health care situation and especially the medical equipment situation, in this case in Ukraine. His analysis supported the reports from other CEEC countries, (see below).

Prof. L. Dalla Palma (Italy), explained the establishment of the Working Group on Cost-Effectiveness within EAR, not least because of the paucity of reports in the radiological press, and progress made towards an initial study, based on work already in progress in one of the sub-specialty societies. He stressed the aims of the Group, to establish methods for measuring the effects of radiology, to develop outcome studies and external evaluation, to adjust radiological techniques to provide optimal benefit to patients, and to provide relevant educational opportunities, and the criteria it applied to achieving those aims.

Mr. Zwerner, (COCIR), pointed out that there are considerable variations between "western" countries in terms of provision of high-tech radiological equipment. He stressed that the costs of radiology, capital and running, were a small proportion of medical costs overall, and that they in turn are a small proportion of GDP. Other problems included demographic changes, which, with an increase in the average age of he population, were unfavourable to the extension of radiology, and an uneven distribution of equipment within countries. He also pointed that the capital costs of CT and MR equipment had decreased during the last 8 years.

Prof. R. Passariello described the position in Italy, with a high proportion of private funding, especially for high-tech equipment, a recent decrease in public funding for radiological equipment, and significant differences between the levels of radiological equipment provision in the North and South of the country. He said that the aims were "efficiency", to do well, "efficacy", to do the right things, and "economy", to do it less expensively. He explained that requests for additional equipment had to be justified in terms of the obsolescence of existing equipment, the organisation and staffing of the department, and number of examinations expected, based on existing demand.

Dr. Pohlenz described the problems in Germany arising from increasing unemployment, increasing health costs and decreasing GDP, and a rise in the number of doctors, not least radiologists. The number of doctors in Germany was 100 000 in 1970, 240 000 in 1996, and was expected to be 280 000 in 1998. There is 20% unemployment amongst the doctors. He explained that in Germany each locality was required to formulate plans for health provision, including x-ray equipment, and to arrange for funding from public and private sources, but noted that in some areas the proposed public funding from public and private sources was not available. A view was emerging that health-care should be considered as an economical factor rather than as a charity, and that hospitals should be allowed to make "profits" and to invest by loans. Considerable increased funding has been promised for former East Germany but it is not adequate. Buyers are encouraged to borrow to make purchases, but not allowed to rent equipment. Dr. Pohlenz recognised the potential ethical problems which might arise.

Speakers from Hungary, Poland, Russia, Belarus, Ukraine, Czechia, Croatia and Georgia described the conditions in their countries. Although there are variations between them, all indicated that the age structure of the population is not significantly different from that in the West, although the expectation of life is somewhat less, and that the major causes of death are the same. Population screening, eg for tuberculosis, is still common. The proportion of GDP spent on "health" varies as it does in Western Europe, but the actual amount per capita is about 25%. Most of the equipment is at least ten years old, and tends to be distributed across many small departments, partly because of

the large areas involved. Problems commonly identified included a traditional structure of large hospitals and long stays, and with respect to radiology, inadequate radiation protection, (though not in Hungary), few intensifiers and high doses of radiation per examination, the need to modernise (and merge) departments, and the lack of high-tech equipment. In addition to the many medical and radiological constraints, it appeared that the infrastructure of the various hospitals and their technical organization are inadequate in many cases and that without a great improvement of these infrastructures the modernisation of radiological departments is going to fail. In Russia in particular an improved teaching programme for radiologists was identified.

Prof. U. Erikson (Sweden) described the evolution of the EAR, (and UEMS), guidelines for radiological education in the undergraduate stage and throughout postgraduate years, specialist training and CME, and strongly recommended the structure to the CEEC, a message which was generally welcomed.

Representatives of the Radiological Industry explained their philosophy and strategy for refining internal working practices and increasing access to CEEC countries, and explained how their methodology could be used by those persons developing health strategies and structures in each country, eg. measures to develop and control quality of service and of radiological image. It was emphasised that high-tech procedures can lead to a reduction in the use of low-tech methods, with earlier/better diagnosis, and that poor or late diagnosis may lead to more expensive care later.

During the Round Table discussions a number of possibilities for progress were brought up:

- local manufacture of components where large numbers are involved because labour costs may be low;
- the substitution of obsolete equipment should preferably be undertaken with digital machines, avoiding the step of new conventional equipment; as analog and digital x-ray machines require the same infrastructure it seems wise to move directly to digital;
- PACS may improve efficiency but also overall results, with the initial rise in costs back to the previous level within 5 years; similarly teleradiology may allow better use of specialists' experience and knowledge;
- companies may be able to develop "Europe Funds" to assist, but it was noted that it could lead to difficulties in selecting equipment, and to perpetuating uneven distribution within countries;
- each country, in both West and East, needs an individual solution, based on guidelines, involving QA, cost-effectiveness and outcome studies, and co-operation between private and public funders, larger and smaller providers, eg. approaches to the World Bank in Croatia and Bulgaria;

In addition, Dr. Bourgade explained some of the difficulties which EU encountered in dealing with some of the matters raised during this Meeting:

- EU can deal only with National Governments, of which only 7 have specific health insurance arrangements, and 8 fund "health" directly from the budget, therefore there is no "Europe Health Fund";
- EU considers a budget for "health" and not only for radiology;
- in many countries, the problem is between the Government and the healthcare providers;
- EU can provide technical help, including analysis of the problem, which should involve health professionals including radiologists, economists and other responsible

persons, and develop an investment programme to attract funding from, eg., the World Bank. An example was a situation in Bulgaria supported by a fund for "emergency care in the community and hospitals", which showed savings within one year, after which an approach was made to the World Bank for continued funding;
- CEEC funding must be distinguished from Third World funding;

In his closing summary, Prof. H. Ringertz thanked all those present and congratulated those presenting papers and contributing to the discussions in what he described as a very useful and forward-looking debate, and suggested that three important messages to be learnt were:

1. the necessity to make detailed strategies when any request for support is mounted, taking into account all costs, including running costs for at least two years, and the possibility of alternative cost methods
2. that it would be worthwhile holding similar meetings later, possibly in individual CEEC countries to consider their peculiar problems, and that EAR would be prepared to arrange such meetings
3. the EAR would be prepared to plan and to arrange such meetings through the ECR Office in Vienna

In conclusion, the Workshop was extremely important in order to focus the majority of items related to investments in Central and Eastern Europe. It appeared that the main problem is the lack of infrastructures in most CEEC countries and the scarcity of data related to their health systems. Almost all speakers underlined the necessity of a deeper knowledge of national needs before funding resources for radiological investments. The post-graduate education of radiologists should be strictly linked with the up-dating of equipment and the improvement of radiological services should be balanced by an equal improvement of the other health services. Finally, EAR demonstrated to be an excellent forum where to discuss, to plan, and to propose further initiatives in this area.

Radiol Oncol 1998; 32(2): 161-3.

Zapora dvanajstnika z žolčnimi kamni (Bouveretov sindrom)

Včev Al, Barbić J, Včev An, Kovačić D, Vegar M

Zapora dvanajstnika z žolčnimi kamni je zelo redko klinično stanje. Običajno jo odkrijemo pri gastrointestinalni endoskopiji ali rentgenski preiskavi zgornjih prebavil. Poročamo o primeru 76-letne ženske z zaporo želodčnega izhoda, nastale zaradi žolčnega kamna. Zaporo smo najprej diagnosticirali z ultrazvožno preiskavo zgornjega abdomna in kasneje potrdili z gastroskopijo. Poskus endoskopske odstranitve žolčnega kamna ni bil uspešen, zato smo kamen odstranili s kirurškim postopkom.

Radiol Oncol 1998; 32(2): 165-9.

Bifemoralna fraktura pri sindromu "trpinčenega otroka "

Klutmann S, Kröger; Bohuslavizki KH, Brenner W, Korff C, Oppermann H-C, Tibow I, Henze E

Poročamo o eno letni deklici, pri kateri je bil postavljen sum na sindrom "trpinčenega otroka". Običajni rentgenogram je pokazal simetrično bifemoralno frakturo blizu rastnih plošč in levostransko parietalno frakturo. Scintigrafija skeleta je odkrila le rahlo simetrično razširitev pripadajočih rastnih področij v aksialnem skeletu v primerjavi s proksimalnimi tibialnimi rastnimi področji, parietalno frakturo ps smo zgrešili. Poudarjamo diagnostične težave in revidiramo kriterije za scintigrafijo skeleta pri sindromu "trpinčenega otroka".

Radiol Oncol 1998; 32(2): 171-85.

Polimerazna verižna reakcija v diagnostiki limfoproliferativnih obolenj

Griesser H

Metode imunohistokemije in molekularne genetike v veliki meri pripomorejo k boljšemu razumevanju limfoproliferativnih obolenj. Pri večini limfomatoznih lezij za diagnozo zadošča že sama morfologija, med tem ko lezije, pri katerih izvor celic in klonalnost nista jasno opredeljeni, zahtevajo dodatne diagnostične preiskave. Morfološko razlikovanje med Hodgkinovim in ne-Hodgkinovimi limfomi, ali med inflamatornimi lezijami in malignim limfomom, je lahko dokaj zapleteno. Nenaključne kromosomske translokacije nam lahko pomagajo pri razpoznavi podskupin z izrazitimi biološkimi značilnostmi. Predmet te pregledne študije so polimerazne verižne reakcije (PCR), ki so dokazale svojo diagnostično uporabnost na določenem celičnem materialu in parafiniziranih tkivnih vzorcih. Učinkovitost PCR pri analizah prerazporejanja T-celičnega receptorja gama in genov težkih verig imunoglobulinov je dobro dokumentirana. Nekatere od znanih translokacij, kot so t(14;18) ali t(2;5), lahko rutinsko ocenjujemo s pomočjo genomske PCR ali PCR obratne transkripcije. Za določanje translokacij bcl-l, bcl-6 in c-myc genov pa so potrebne bolj zapletene in sofisticirane metode s PCR. Poleg svojega neposrednega diagnostičnega pomena imajo molekularne genske analize s pomočjo PCR še to prednost, da v povezavi s citomorfologijo in imunofenotipizacijo omogočajo določanje novih kriterijev v diagnostiki limfomov.

Radiol Oncol 1998; 32(2): 187-91.

In vivo elektroporacija sečnega mehurja miši

Veranič P, Jezernik K, Čemažar M, and Serša G

Celična membrana je poglavitna prepreka za vključevanje različnih snovi v celice. Plazmalema površinskih celic urotelija sečnega mehurja je pri sesalcih močno odebeljena in mehansko stabilna, tako da preprečuje vstopanje zunajceličnih molekul v citosol. Ena od metod, ki omogočajo prehajanje citokemičnih označevalcev v celico, je tudi elektroporacija. Na urotelijskih celicah elektroporacija še ni bila opisana. Da bi ugotovili ustreznost metode za vstopanje označevalcev v celice, smo sečne mehurje miši izpostavili osmim zaporednim električnim pulzom z amplitudo 720 V in 1040 V. Uporabili smo označevalce z različnimi molekulskimi težami: tripansko modrilo, TRITC-faloidin in FITC-protitelo (IgG). Rezultati so pokazali, da elektroporacija omogoči vstopanje označevalcev skozi odebeljeno plazmalemo urotelijskih celic, zato jo lahko uporabljamo kot dodatno metodo pri označevanju celičnih sestavin.

Radiol Oncol 1998; 32(2): 193-200.

Desmuramilni analog LK-404 oslabi s ciklofosfamidom inducirano apoptozo celic iz kostnega mozga in vranice

Kostanjšek R, Kuralt P, Malovrh T, Škoberne M, Štalc A, Kotnik V

Ciklofosfamid je citostatik, ki poleg tumorskih celic ubija tudi imunske celice. Smrt povzroči z indukcijo apoptoze. V prispevku poročamo o in vitro in in vivo smrtonosnem vplivu ciklofosfamida na kostni mozeg in na vranične celice. Z večanjem koncentracije in daljšanjem časa inkubacije s ciklofosfamidom se je povečevala moč apoptoze pri obeh načinih tretiranja. V nadaljevanju raziskave smo s ciklofosfamidom inducirano apoptozo poskušali zavreti ali pa jo povsem ustaviti. Zato smo dodajali v celično kulturo kostnega mozga ali vraničnih celic desmuramilni analog LK-404. LK-404 ima imunomodulatorne lastnosti in med drugim vpliva tudi na razmnoževanje in zorenje imunskih celic v kostnem mozgu in vranici. Če smo gojili kostni mozeg ali vranične celice istočasno s ciklofosfamidom in LK-404, nismo opazili nikakršnih sprememb v jakosti apoptoze. Če pa smo oboje celice najprej inkubirali uro in pol s substanco LK-404, nato pa dodali ciklofosfamid, se je moč apoptoze zaznavno zmanjšala. Ugotovitev zbuja misel, da lahko desmuramilni analog LK-404 zavaruje celice kostnega mozga in vranice pred s ciklofosfamidom inducirano apoptozo.

Radiol Oncol 1998; 32(2): 201-5.

Vzroki za nastanek raka testisov

Kovač V

Vzroki za nastanek raka testisov so, kakor pri večini drugih vrstah raka, neznani. Možne vzroke iščejo s proučevanjem tistih skupin moških, kjer je incidenca te bolezni zvišana. Tako opisujejo družinsko obliko raka testisov, navajajo vpliv onkogenov, posebno pa izpostavljajo predispozicijske dejavnike, ki jih predstavljajo kongenitalne anomalije urogenitalnega trakta. Anomalija, ki je v največji meri povezana z nastankom raka testisov, je kriptorhisem in ker pogostnost kriptorhisma v svetu raste, naj bi bil tudi to glavni vzrok za povečano incidenco raka testisov.

Posebno vlogo pri nastanku te vrste raka pripisujejo atrofiji germinalnega epitelija. V novejšem času pa intenzivno proučujejo vpliv estrogenov iz okolja, ki lahko okvarjajo spermatogenezo in verjetno povzročajo večjo incidenco prirojenih anomalij urogenitalnega trakta.

Zanimiv je vpliv socialnih dejavnikov, medtem ko vpliv traume na nastanek raka testisov ni dokazan, lahko pa bi bila poškodba testisov sovzrok za nastanek raka ob drugih preeksistenčnih vzrokih.

Radiol Oncol 1998; 32(2): 207-11.

Limfotropično barvanje "stražarskih" bezgavk pri raku dojke S čem, kdaj, kako

Baychev G, Delijsky T, Penkova R, Stojanov R

Namen študije je bil definirati način izbire pravega barvila za označevanje "stražarskih" bezgavk pri raku dojke. Pre ali intraoperativno smo uporabili barvila kot Metilen Blue, Drimaren briliant Blue, Patent Blue V okoli tumorja pri 135 bolnicah. Za ojačanje limfotropizma smo pri 92 bolnicah uporabili Gelatin, Alvezin, Haemodex ali HAES raztopine kot nosilce barvila. Volumen se je spreminjal od 1,0 do 3,0 ml, v 25% primerov smo predhodno uporabili Hilazo za povečanje absorbcije. V študijo je bilo vključenih tudi 29 bolnic s preoperativno kemoterapijo z Mitoxantronom. Citostatično modro barvo smo uporabili za identifikacijo prvih limfnih žil in betgavk. Najpogosteje smo vizualizacijo dosegli z Mitoxantronom (80%), Patent Blue V (70%) in kombinacijo Drimaren Briliant Blue z Haemodexom (57%).

Radiol Oncol 1998; 32(2): 213-20.

Rak ščitnice: pooperativna radioterapija

Mayer R, Stueckelschweiger GF, Preidler HW, Pakisch B, Langsteger W, Oechs A, Prettenhofer U, Hackl A

Naredili smo retrospektivno analizo bolnikov s ščitničnim rakom, ki so prejeli pooperativno teleradioterapijo in visokodozni jod.
Med leti 1982 in 1993, smo imeli bilo 69 bolnikov s štirimi histološkimi tipi ščitničnega raka (folikularni, n=17; papilarni, n=39; medularni, n=7; anaplastični, n=6) zdravljenih kirurško, z ablativnim radiojodom in z radioterapijo. Indikacije za adjuvantno radioterapijo so bile izvenžlezna tumorska infiltracija (stadij pT4) in/ali neradikalna resekcija in/ali obsežna prizadetost bezgavk ali težka kirurška ekscizija ponavljajočih se lokalnih recidivov. Radioterapijo smo izvajali s paralelnima opozitnima poljema (50 Gy, 2 Gy) z "mini- mantle" tehniko, temu pa je sledil še elektronski "boost" (10 do 14 Gy, 2 Gy) s sredinskim voščenim bolusom.
Pet letno preživetje je bilo 82,6%, pet letno preživetje brez bolezni je bilo 92,1% (povprečna opazovana doba 56 mesecev, od 4 do 146 mesecev). Opazili smo statistično pomembne razlike med prilagojenim preživetjem in parametri kot so histologija ($p< 0,00001$), prizadetost vratnih bezgavk ($p< 0,002$), metastaze ob odkritju ($p< 0,001$) in starost ($p<0.03$). Nismo pa zabeležili resnih poznih zapletov posebej poškodb hrbtenice in sapnika.
Kot kaže, je pooperativna visokodozna radioterapija (60 d0 64 Gy), v primerih diferenciranih ščitničnih tumorjev kombinirana z visokodozno terapijo z radiojodom, učinkovito orodje za sterilizacijo mikroskopske in makroskopske rezidualne bolezni in jo lahko varno uporabljamo ob pomoči modernih terapevtskih tehnik.

Radiol Oncol 1998; 32(2): 221-4.

Rekonstrukcija defektov na glavi in vratu z režnji velike pektoralne mišice

Yildirim E, Turanli M, Sancaktar S, Berberoglu U

Med letoma 1991 in 1995 smo v Onkološki bolnišnici v Ankari Uporabili Miokutani reženj velike pektoralne mišice za rekonstrukcijo defektov na glavi in vratu v 26 primerih in osteomiokutani reženj prav tako velike pektoralne mišice v enem primeru. Resekcijo mandibule smo opravili pri 13 pacientih. Med temi jih je bilo 9 s segmentno mandibulektomijo, 7 z marginalno in eden s hemimandibulektomijo. Perioperativna smrtnost je bila 3,7%. Do ponovitve bolezni v področju režnja je prišlo pri 6 bolnikih, do deformacije mandibule pa tudi pri 6 bolnikih. Kot zaključek lahko rečemo, da je osteomiokutani reženj velike pektoralne mišice primeren za takojšnje rekonstrukcije v področju glave in vratu.

Notices

Notices submitted for publication should contain a mailing address, phone and/or fax number and/or e-mail of a contact person or department.

Lung Cancer

August 9-12, 1998.

The symposium "Clinical Implications of Molecular Epidemiology of Human Lung Cancer" will be held in Oslo, Norway.

Contact Conference Secretariat, Help Arrangement-Service AS, P.O. Box 527, N-1301 Sandvika, Norway; or call +47 67 56 90 12; or fax +47 67 56 44 80. E-mail: chaskim@online.no. Internet http://www.stami.no/lcs

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Urology

August 12-13, 1998.

The ESO training course "Genito-urinary tract tumours" will take place in Sao Paulo, Brazil.

Contact ESO Latin America, Dr.A.Frasson, Ave. Ipiranga 6690, Porto Alegre, Brazil; or call +55 51 3392 709; or fax +55 51 3392 709.

Breast cancer

August 14, 1998.

The ESO training course "Breast Cancer, Diagnosis and Treatment" will be offered in Rio de Janeiro, Brazil.

Contact ESO Latin America, Dr.A.Frasson, Ave. Ipiranga 6690, Porto Alegre, Brazil; or call +55 51 3392 709; or fax +55 51 3392 709.

Radiotherapy

August 14, 1998.

The ESO training course on curiterapie will be offered as course pre-congress of the UICC in Rio de Janeiro, Brazil.

Contact ESO Latin America, Dr.A.Frasson, Ave. Ipiranga 6690, Porto Alegre, Brazil; or call +55 51 3392 709; or fax +55 51 3392 709.

As a service to our readers, notices of meetings or courses will be inserted free of charge.
Please send information to the Editorial office, Radiology and Oncology, Vrazov trg 4, 1000 Ljubljana, Slovenia.

Oncology

August 24-28, 1998.

The "17th UICC International Cancer Congress" will be held in Rio de Janeiro, Brazil.

Contact c/o Congrex do Brasil, Rua do Ouvidor, 60 grupo 413, 20010-030 Rio de Janeiro, RJ Brazil; or call +55 21 224 6080; or fax +55 21 231 1492.

Radiotherapy

August 30- Septembr 3 1998.

The "ESTRO Teaching Course on Physics for Clinical Radiotherapy" will take place in Leuven, Belgium.

Contact ESTRO Office: Av. Mounier 83-1200 Brussels, Belgium; or call +32 2 775 93 40; or fax +32 2 779 54 94. E-mail: zdravka.dimitrova@estro.be

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Oncology

September, 1998.

The EORTC course "One Day Introduction to EORTC Trials" will be held in Brussels, Belgium.

Contact EORTC Education office, Av. E. Mounier 83/11, 1200 Brussels, Belgium; or call +32 2 772 4621; or fax +32 2 772 6233. Internet: http/www.eortc.be

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Radiation Physics

September, 1998.

The ESTRO teaching course "Radiation Physics for Clinical Radiotherapy" will be offered in Leuven, Belgium.

Contact the ESTRO office, Av. E. Mounierlaan, 83/4, B-1200 Brussles, Belgium; or call +32 2 775 9344; or fax +32 2 779 5470. E-mail: germaine.heeren@estro.be

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Haematology

September, 1998.

The ESO training course "Biology and Treatment of Plasma Cell Dyscrasias" will be offered in Athens, Greece.

Contact ESO Balkans and Middle East Office, Egnatia Epirus Foundation, 7A Tzavella St., 453 33 loannina, Greece; or call +30 651 72315/76992; or fax +30 651 36695.

Palliative care

September 8-9, 1998.

The ESO training course will be held in Costa Rica.

Contact ESO Latin America, Dr.G.Farante, Viale Beatrice d'Este 37, 20122 Milan, Italy; or call +39 2 5831 7850; or fax +39 2 5832 1266. E-mail: comprevtum@bbs.infosquare.it

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Prostate cancer

September 10-12, 1998.

The ESO advanced course "Prostate Cancer" will be offered in Milan, Italy.

Contact European School of Oncology, Viale Beatrice d'Este 37, 20122 Milan, Italy; or call +39 2 5831 7850; or fax +39 2 5832 1266. E-mail: comprevtum@bbs.infosquare.it

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Breast cancer

September 11-12, 1998.

The ESO training course will be held in Montevideo, Uruguay.

Contact ESO Latin America, Dr.G.Farante, Viale Beatrice d'Este 37, 20122 Milan, Italy; or call +39 2 5831 7850; or fax +39 2 5832 1266. E-mail: comprevtum@bbs.infosquare.it

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Oncology

September 12-13, 1998.

The ESO training course "Sarcomas and Malignant Melanoma" will take place in Athens, Greece.

Contact ESO Balkans and Middle East Office, Egnatia Epirus Foundation, 7A Tzavella St., 453 33 loannina, Greece; or call +30 651 72315/76992; or fax +30 651 36695.

Lung Cancer

September 13-16, 1998.

The "5th Central European Lung Cancer Conference" will take place in Prague, Czech Republic.

Contact 5th Central European Lung Cancer Conference, Conference secretariat, Czech Medical Association J.E. Purkyne, P.O. Box 88, Sokolska 31, 120 26 Prague 2, Czech Republic; or call +420 2 296 889, +420 2 297 271; or fax +420 2 294 610, +420 2 2421 6836. E-mail: lon@czechmed.anet.cz

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Breast cancer

September 14-15, 1998.

The ESO training course will be held in Santiago, Chile.

Contact ESO Latin America, Dr.G.Farante, Viale Beatrice d'Este 37, 20122 Milan, Italy; or call +39 2 5831 7850; or fax +39 2 5832 1266. E-mail: comprevtum@bbs.infosquare.it

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Computer tomography

September 16-19, 1998.

The postgraduate course "Spiral CT of the Thorax" will be offered in Lille, France.

Contact the Secretarial office, Department of Radiology, Pr Remy, Hospital Calmette, Boulevard Jules Leclerc, 59037 Lille Cedex, France; fax +33 3 2044 4720; E-mail: mremy-jardin@chru-lille.fr

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Radiotherapy and Oncology

September 19-24, 1998.

The "17th Annual ESTRO Meeting" will be offered in Edinburgh, UK.

Contact the ESTRO office, Av. E. Mounierlaan, 83/4, B-1200 Brussles, Belgium; or call +32 2 775 9344; or fax +32 2 779 5470. E-mail:germaine.heeren@estro.be

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Radiotherapy

September 19-24, 1998.

The "4th Postgraduate Teaching Course", organised by ERTED (European Radiotherapy Technologist Education Development Group) will be held in Edinburgh, UK, at the time of the 17th Annual ESTRO Meeting.

Contact the ESTRO office, Av. E. Mounierlaan, 83/4, B-1200 Brussles, Belgium; or call +32 2 775 9344; or fax +32 2 779 5470. E-mail:germaine.heeren@estro.be

Medical oncology

September 24-26, 1998.

The ESO advanced course on medical oncology will be offered in Milan, Italy.

Contact European School of Oncology, Viale Beatrice d'Este 37, 20122 Milan, Italy; or call +39 2 5831 7850; or fax +39 2 5832 1266. E-mail: comprevtum@bbs.infosquare.it

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Radiotherapy and Oncology

September 25-28, 1998.

The "19th Annual ESTRO Meeting" will be held in Prague, Czech Repub-lic.

Contact the ESTRO office, Av. E. Mounierlaan, 83/4, B-1200 Brussles, Belgium; or call +32 2 775 9344; or fax +32 2 779 5470. E-mail:germaine.heeren@estro.be

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Haematology

September 28-30, 1998.

The "3rd Educational Forum on Leukaemia and Haematological Malignancies" will be held in Bergamo, Italy.

Contact European School of Oncology, Viale Beatrice d'Este 37, 20122 Milan, Italy; or call +39 2 5831 7850; or fax +39 2 5832 1266. E-mail: comprevtum@bbs.infosquare.it

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Radiotherapy

October, 1998.

The ESTRO teaching course "Evidence Based Radiation Oncology: Principles and Methods" will take place in Izmir, Turkey.

Contact the ESTRO office, Av. E. Mounierlaan, 83/4, B-1200 Brussles, Belgium; or call +32 2 775 9344; or fax +32 2 779 5470. E-mail:germaine.heeren@estro.be

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Radiobiology

October, 1998.

The ESTRO teaching course "Basic Clinical Radiobiology" will be held in Como, Italy.

Contact the ESTRO office, Av. E. Mounierlaan, 83/4, B-1200 Brussles, Belgium; or call +32 2 775 9344; or fax +32 2 779 5470. E-mail:germaine.heeren@estro.be

Surgical oncology

October 1-3, 1998.

The ESO advanced course "Breast Reconstructive and Cancer Surgery II" will take place in Milan, Italy.

Contact European School of Oncology, Viale Beatrice d'Este 37, 20122 Milan, Italy; or call +39 2 5831 7850; or fax +39 2 5832 1266. E-mail: comprevtum@bbs.infosquare.it

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Oncology

October 7-9, 1998.

The ESO training course "Innovation in Cancer Management" will be offered in Cairo, Egypt.

Contact ESO Balkans and Middle East Office, Egnatia Epirus Foundation, 7A Tzavella St., 453 33 Ioannina, Greece; or call +30 651 72315/76992; or fax +30 651 36695.

Oncology

October 8-9, 1998.

The ESO training course "Rare Tumours: Diagnosis and Treatment" will be held in Sofia, Bulgaria.

Contact ESO Balkans and Middle East Office, Egnatia Epirus Foundation, 7A Tzavella St., 453 33 Ioannina, Greece; or call +30 651 72315/76992; or fax +30 651 36695.

Oncology

October 15-21, 1998.

The ESO training course "Liver Pathology-Oncology" will be offered in Ioannina, Greece.

Contact ESO Balkans and Middle East Office, Egnatia Epirus Foundation, 7A Tzavella St., 453 33 Ioannina, Greece; or call +30 651 72315/76992; or fax +30 651 36695.

Haematology and Oncology

October 19-21 1998

The "3rd Educational Forum on Leukaemias: State of art and hot issues" will be held in Bergamo, Italy

Contact European School of Oncology, Viale Beatrice d'Este 37, 20122 Milan, Italy; or call +39 2 5831 7850; or fax +39 2 5832 1266. E-mail: comprevtum@bbs.infosquare.it

Radiation Oncology

October 19-22, 1998.

The "Annual Meeting of American Society for Therapeutic Radiology and Oncology ASTRO" will take place in Phoenix, Arizona, USA,

Contact Vicky Carroll, ASTRO office, 1891 Preston White Drive, Reston, VA 22091, USA; or call +1 703 716 7588; or fax +1 703 476 8167.

Laser medicine & surgery

October 21-25, 1998.

The "7th Congress of Asian Pacific Association for Laser Medicine & surgery" and the "10th international YAG Laser Symposium" will be offered in Ho Chi Minh City, Vietnam.

Contact Dr. Ha Viet Hien, Secretariat officer, 109A Pasteur St, Dist 1, Ho Chi Minh City, Vietnam; or call +84 8 829 9322/ 824 1958; or fax +84 8 824 1959. E-mail: biomed@bdvn.vnd.net

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Colorectal cancer

October 22-24, 1998.

The ESO advanced course "Digestive Tract: Colorectal Cancer" will be held in Milan, Italy.

Contact European School of Oncology, Viale Beatrice d'Este 37, 20122 Milan, Italy; or call +39 2 5831 7850; or fax +39 2 5832 1266. E-mail: comprevtum@bbs.infosquare.it

Haematology and oncology

October 25-28, 1998.

The Annual Meeting of German and Austrian Association of Haematology and Oncology will be offered in Frankfurt, Germany.

Contact Prof. Dr. Dieter Hoelzer, Universitaetklinik Frankfurt, Medizinische Klinik III, Theodor Stern Kai 7, 60590 Frankfurt, Germany; or call +49 39 6301 5194; or fax +49 69 6301 7324; e-mail: hoelzer@em.uni-frankfurt.de

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Paediatric Oncology

October 27-31, 1998.

The S.I.O.P. teaching course will take place in Moscow, Russia.

Call P.A. Voute +31 20 566 5655; or fax +31 20 691 2231.

Lung cancer

October 31 - November 4, 1998.

The 3rd interantional congress on lung cancer will be offered in Rhodes, Greece.

Contact Congress Secretariat of the 3rd interantional congress on lung cancer, Amphitrion Congress Organising Bureau, 7, Sygrou Avenue, 117 43 Athens, Greece; or call +30 1 924 9701; or fax +30 1 924 9836 / 924 9671. E-mail: amphitrion@travelling.gr

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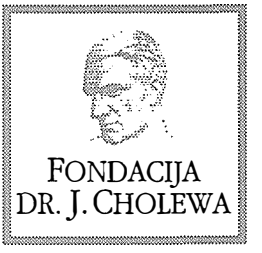

Activity of "Dr. J. Cholewa" foundation for cancer research and education - report for the second quarter of 1998

In the first and the second quarter of 1998 the "Dr. J. Cholewa" foundation for cancer research and education sought to ensure the continuation of its activities in the view of overcoming ever presenting financial constraints as conditioned by the general level of the economic activity in the republic of Slovenia. With this in mind, better coordination with other similar institutions in Slovenia has been of paramount importance.

The Foundation continues to support regular publication of "Radiology and Oncology" international scientific journal, and the regular publication of the "ESO Challenge", the newsletter of the European school of Oncology, both being published and edited in Ljubljana, Slovenia. It actively supported the organisation of the first education "Oncological weekend" meeting held in 1998, that was intended to all in the medical profession interested in problems connected with pediatric oncology. The meeting was held in the city of Postojna and has since been regarded as very successful by its participants and organizers. The preparation proceedings for the traditional Hepatobiliary School in Ljubljana are now gathering momentum, and the Foundation hopes to provide its modest contribution.

For the 1998 the Foundation also plans to continue to provide grants for the various European School of Oncology courses, research and educational grants for the study in Slovenia and abroad, to provide support for educational and scientific meetings and symposia, and to support publishing and editorial activity from the various fields of oncology in Slovenia.

It can thus be seen that the " Dr. J. Cholewa" Foundation for Cancer Research and Education continues to pursue its stated goals, as defined by its statute and recent meetings of the Board of directors and the Assembly of the Foundation.

Tomaž Benulič, MD
Borut Štabuc, MD, PhD
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THE 5th ANNUAL MEETING OF THE EUROPEAN SOCIETY OF MUSCULOSKELETAL RADIOLOGY

BLED, SLOVENIA
OCTOBER 30-31, 1998.

The fifth Annual Meeting of the European Society of Musculoskeletal Radiology (ESSR) will be held in Bled, Slovenia, October 30-31, 1998.

Our young society celebrates its fifth anniversary, having a short but very seccessful history. The society was founded on March 20th, 1993 in Bonn, Germany by thirteen representatives from various European countries. This was a modest beginning of the ESSR, which, today, is an established society with 218 members from 29 European countries. Our main task is to promote musculoskeletal radiology in Europe, with a particular emphasis on education and research. One of the most important activitiesof the ESSR is the organization of our annual meeting which is an opportunity to share research experience and to refresh the knowledge of diferent areas in musculoskeletal radiology.

We have a great honour and pleasure to organize the meeting in Slovenia for the first time. Two refresher courses and fourteen parallel scientific sessions are scheduled. Introductory lectures will be given by distinguished musculoskeletal radiologists. Young colleagues are encouraged to present the results of their research work.

On behalf of the Organizing Committee I cordialy invite you to attend the Fifth Annual Meeting of the ESSR.

V, Jevtic, President of the ESSR

INFORMATION:
Professor V. Jevtic, M.D.
Clinical Radiology Institute
University medical Centre
Zaloška 7
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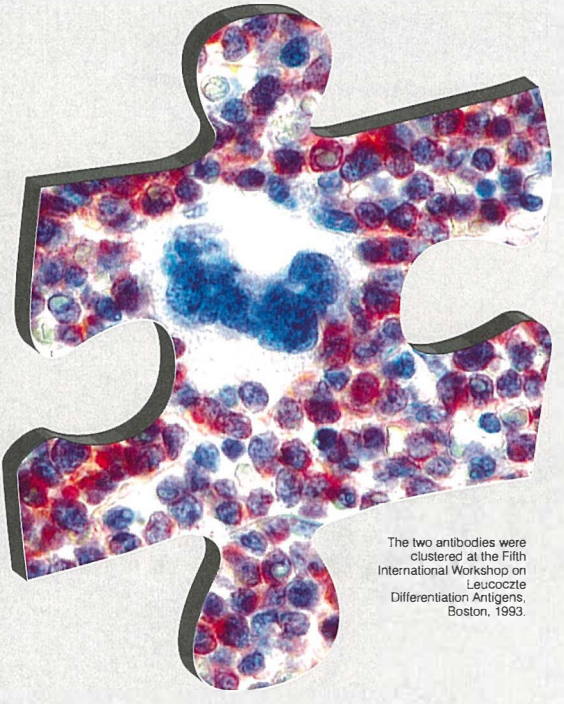

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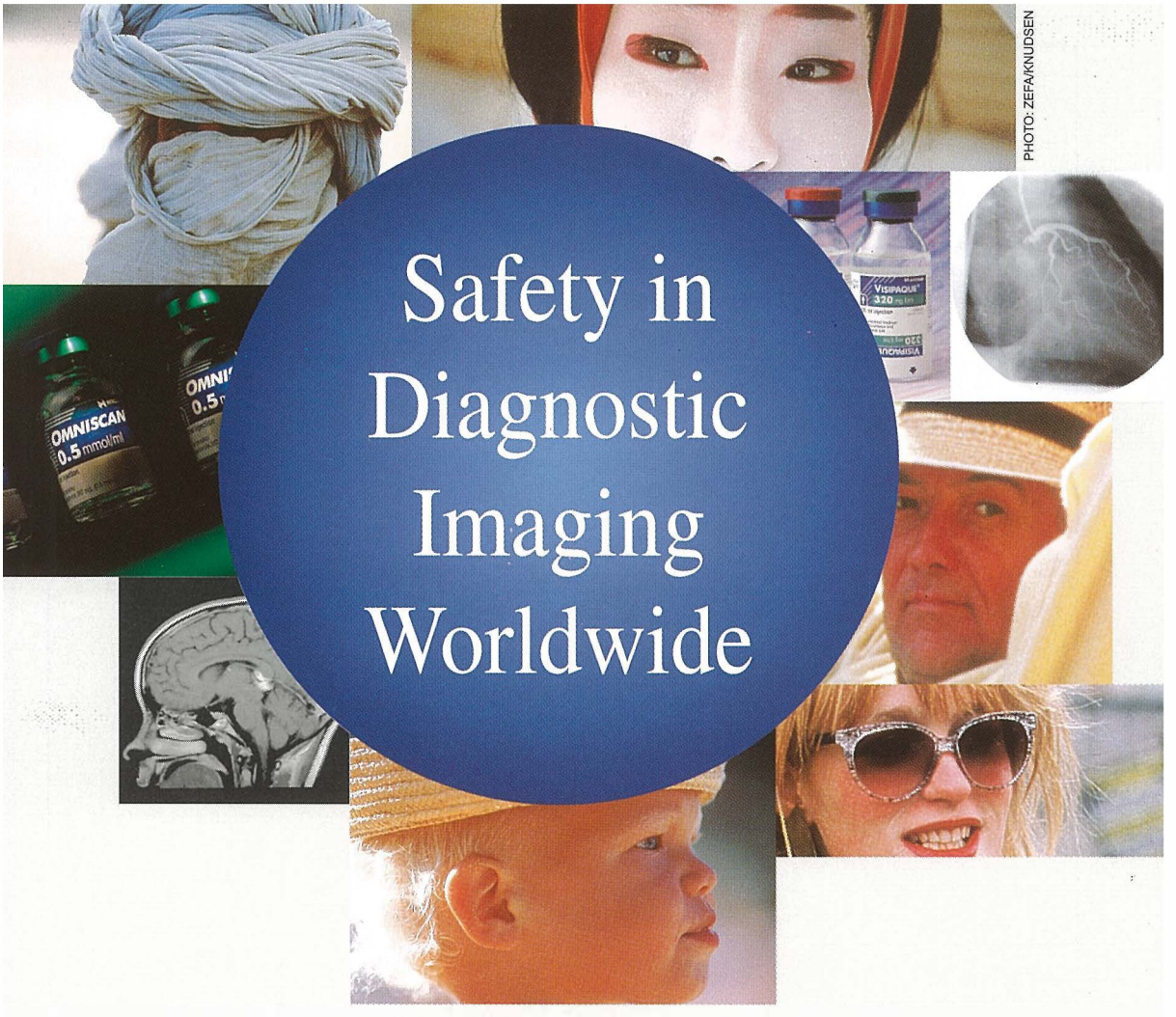

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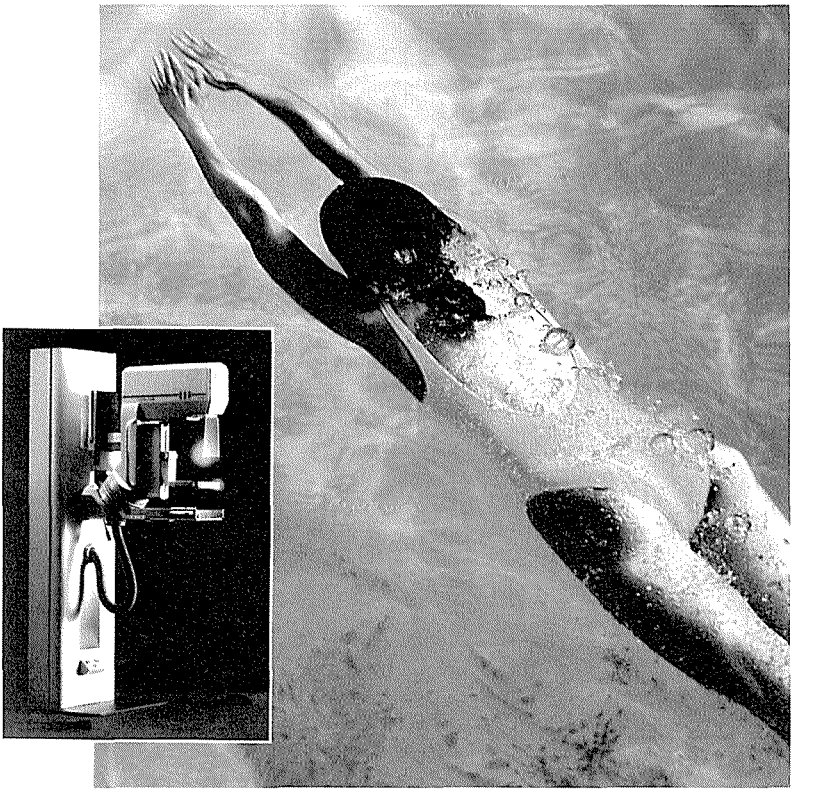

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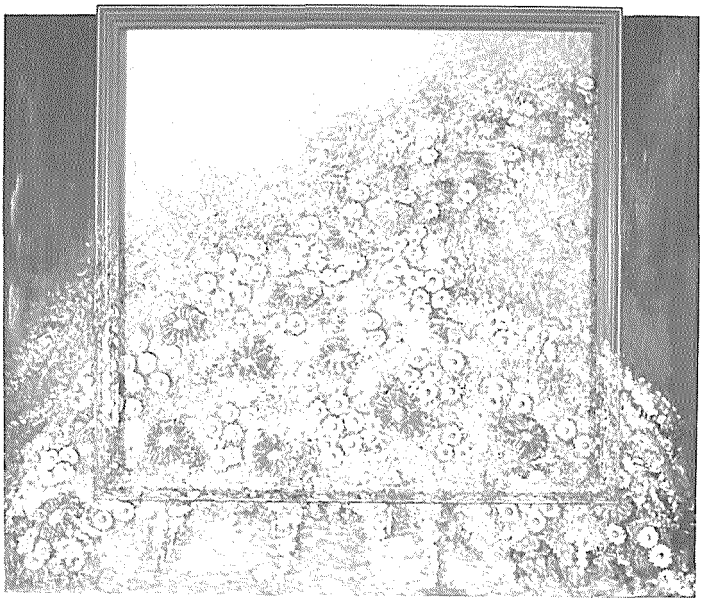

– centralno delujoči analgetik za lajšanje zmernih in hudih bolečin

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– tramadol je registriran tudi pri ameriški zvezni upravi za hrano in zdravila (FDA)

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Results should be presented clearly and concisely without repeating the data in the tables and figures. Emphasis should be on clear and precise presentation of results and their significance in relation to the aim of the investigation.

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