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Modulation of the peripheral immune system after low-dose radon spa therapy: Detailed longitudinal immune monitoring of patients within the RAD-ON01 study

Paul F. Rühle, Roland Wunderlich, Lisa Deloch, Claudia Fournier, Andreas Maier, Gerhart Klein, Rainer Fietkau, Udo S. Gaipl, and Benjamin Frey

1Department of Radiation Oncology, Universitätsklinikum Erlangen, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany, 2Research Unit of Radiation Cytogenetics, Helmholtz Center Munich, Neuherberg, Germany, 3GSI Helmholtzzentrum für Schwerionenforschung, Darmstadt, Germany, and 4Practice for Cardiology, Bad Steben, Germany

Abstract

The pain-relieving effects of low-dose radon therapies on patients suffering from chronic painful inflammatory diseases have been described for centuries. Even though it has been suggested that low doses of radiation may attenuate chronic inflammation, the underlying mechanisms remain elusive. Thus, the RAD-ON01 study was initiated to examine the effects of radon spa therapy and its low doses of alpha radiation on the human immune system. In addition to an evaluation of pain parameters, blood was drawn from 100 patients suffering from chronic painful degenerative musculoskeletal diseases before as well as 6, 12, 18, and 30 weeks after the start of therapy. We verified significant long-term pain reduction for the majority of patients which was accompanied by modulations of the peripheral immune cells. Detailed immune monitoring was performed using a multicolor flow cytometry-based whole blood assay. After therapy, the major immune cells were only marginally affected. Nevertheless, a small but long-lasting increase in T cells and monocytes was observed. Moreover, neutrophils, eosinophils and, in particular, dendritic cells were temporarily modulated after therapy. Regarding the immune cell subsets, cytotoxic T and NK cells, in particular, were altered. However, the most prominent effects were identified in a strong reduction of the activation marker CD69 on T, B, and NK cells. Simultaneously, the percentage of HLA-DR+ T cells was elevated after therapy. The RAD-ON01 study showed for the first time a modulation of the peripheral immune cells following standard radon spa therapy. These modulations are in line with attenuation of inflammation.

Keywords

Radon spa, immune monitoring, chronic painful musculoskeletal diseases, attenuation of inflammation, immune system

Introduction

The beneficial effects of low-dose radiation therapy (LD-RT) using X-rays for patients suffering from chronic pain were already being described at the end of the nineteenth century [1]. The reports of pain reduction after bathing in certain natural springs can be found from even earlier periods. In the early twentieth century, the radioactive noble gas radon was found in many of these springs. Today, small doses of about 0.2–0.5 mSv of alpha radiation emitted by radon are believed to be responsible for analgesic and anti-inflammatory effects [2]. Clinical improvements in inflammatory and degenerative diseases after exposure to low doses of radiation range from long-term pain reduction to complete analgesia [2–8]. Nevertheless, the underlying mechanisms are still widely unknown [9].

An acute inflammatory response is a highly coordinated and protective process. It is accompanied by the five macroscopic signs pain, heat, swelling, redness, and loss of function, which reflect vasodilation and extravasation of immune cells into the target tissue. However, when this acute response fails to be resolved, chronic inflammation may persist. Normally, the extent of chronic inflammation is lower than during an acute response. Nevertheless, the affected patient suffers from the same macroscopic signs, whereby pain and loss of function may be the most prominent symptoms in painful degenerative diseases. Rheumatoid arthritis (RA) is also characterized by chronic inflammation, here of the synovium.

It is widely accepted that the immune system can be modulated by radiation [10,11], and several pre-clinical observations prove that low doses of radiation attenuate...
existing inflammation or the inflammatory phenotype of immune cells [12–17]. However, these investigations were mostly based on X-irradiation as this is prevalent in clinical applications, especially in the treatment of local chronic inflammatory diseases [18]. In contrast, radon therapy is also applied for chronic multi-morbid disorders, but is only available in health spas with natural occurrences of this noble gas. Long-term effects on pain reduction have been observed for RA in particular [2]. It has been proposed that the immune system is involved in this radon-dependent pain reduction [19–21] and the Multidisciplinary European Low Dose Initiative (MELODI) has suggested interconnecting radiation research and immunology [22]. However, detailed longitudinal analyses of the immune status of patients during radon spa therapy have not been conducted so far.

Consequently, the RAD-ON01 study presented here was initiated to explore for the first time the impact of low doses of alpha irradiation on the peripheral immune system during standard radon spa therapy. The peripheral blood of 100 patients with chronic painful degenerative musculoskeletal disorders was subjected to detailed immunophenotyping before and after radon spa therapy including follow-up for 7 months. For this purpose, we developed a modular assay for detailed immunophenotyping of peripheral human whole blood samples by multicolor flow cytometry [23]. It allows the characterization of 34 different cell subsets covering all major immune cells such as T, B, and NK cells, as well as dendritic cells (DCs), monocytes, neutrophils, eosinophils, basophils, and circulating hematopoietic stem cells. Furthermore, the activation status of these cells was evaluated.

Methods

Study design and patients

The RAD-ON01 study was a prospective and explorative trial with 103 patients suffering from chronic painful musculoskeletal disorders of the spine and/or joints (ethical approval: BLAK #12131). Since two patients only attended the examination before therapy and one patient could not attend the final examination, all displayed data refer to the 100 patients for whom all immunophenotyping data were available. The patient characteristics are summarized in Table 1. All patients underwent radon spa therapy in March 2013 at the certified health resort Bad Steben (Bavaria, Germany) and were followed up for 30 weeks. A prerequisite for inclusion in the RAD-ON01 study was that all patients had a pain anamnesis of at least 1 year and had undergone previous drug treatments and/or physiotherapy without lasting success. Only patients living in close proximity to Bad Steben were recruited to decrease the impact of environmental differences and to exclude placebo effects related to holiday benefits.

The study design was based on standard radon spa applications and in particular on former radon studies that had been conducted in Bad Steben [24]. Thus, radon spa therapy was given as a series of nine baths (each 20 min, 34 °C) in natural radon spring water (600–1200 Bq/l) over 3 weeks (3 baths per week). The cumulative effective dose of radiation received in this radon spa treatment was estimated to be approximately 0.3 mSv [25]. Complementary estimations of the radiation dose that reaches the tissue during radon spa therapy are currently being produced and examined in the GREWIS (genetic risks and the anti-inflammatory action of ionizing radiation) research project.

A placebo group could not be included in the RAD-ON01 study because of legal issues regarding radiation protection. Even though patients are allowed to undergo radon spa therapy after prescription, the situation is different from a legal perspective in a study including a placebo group where only some patients would be sent into the radon spa. Nevertheless, we are currently working on setting up a RAD-ON02 study that will include a temporary placebo group (cross-over design).

Examination of the patients and follow-up

This radon spa therapy was applied as monotherapy. In order to estimate external influences, participants’ residential situation, previous radon therapies and undesired but potential medication intake or other treatments were documented.

The patients were examined five times on-site: before therapy as well as at 6, 12, 18, and 30 weeks after the start of therapy. They were examined for different pain and cardiovascular parameters (not outlined here). The trigger point measurements by dolorimetry were only performed until week 18 due to fixed follow-up care agreements.

Measurement of pain parameters

The evaluation of individual pain perception was performed using visual analog scales (VAS) ranging from 0 (no pain) to 10 (worst pain imaginable) that were filled out by every patient. To obtain additional objective pain parameters, dolorimetry was also performed. Therefore, eight pressure points were defined according to common practice and applicable pressure was measured to evaluate each patient’s individual pain sensitivity [24]. Moreover, all patients evaluated their individual pain development retrospectively at the end of the study using VAS.

Immunophenotyping: data acquisition and analysis

Additionally, at all five time points, blood was drawn and transported to Universitätsklinikum Erlangen within 3 h. All immunological investigations were performed here in the Laboratory of Radiation Immunobiology at the Department of Radiation Oncology. Upon arrival the blood was immediately
processed for multicolor flow cytometric analyses by the in-house established detailed immunophenotyping of blood (DiO-B) assay [23]. Essentially, this DiO-B assay comprises 11 staining panels requiring about 2.0 ml of whole blood. For each staining, 100 μl of blood were incubated with freshly prepared antibody mix. After incubation for 25 min in the dark at room temperature, erythrocytes were lysed and leukocytes were fixed in an automated three-step process using the TQ-Prep Workstation (Beckman Coulter, Krefeld, Germany). All samples were then washed twice with phosphate-buffered saline (PBS) and kept on ice in PBS containing 1% paraformaldehyde until measurement. For acquisition, the Gallios flow cytometer (Beckman Coulter) with three lasers in standard filter configuration was used. All 500 blood samples were processed by trained staff using previously established standard operating protocols, constant cytometer settings and fresh antibody mixes.

The data obtained were analyzed using the Kaluza analysis software (v.1.2; Beckman Coulter). Since strong variations in blood sample conditions were detected at the fourth time point (18 weeks), most likely due to the high summer temperatures in July 2013, we excluded this time point from analysis in advance. The percentages of all cell subsets were then calculated in relation to total leukocytes or their respective major cell type (e.g. CD4+ T helper cells out of all CD3+ T cells) using MS Excel (Microsoft, Redmond, WA).

**Statistical analysis**

Statistical analyses were carried out using the IBM SPSS Statistics software (v.21.0.0.0, International Business Machines, Armonk, NY). For the immunophenotyping, the paired t-test was used for statistical comparison of the data from time points 6, 12, and 30 weeks after treatment with the data from before therapy. For the pain parameters, all statistical analyses were also performed versus week 0 (before start of therapy) using the Friedman test followed by Wilcoxon correction for the VAS data and ANOVA or ANOVA with repeated measurements for the dolorimetry data of maximal and mean pressure points, respectively.

**Results**

**Pain relief**

The RAD-ON01 study demonstrated long-lasting pain reduction after radon spa therapy for the majority of patients (87%). Evaluation of the VAS, which was filled out by all patients at all time points, revealed a long-lasting and significant reduction of pain for the complete observation period (Figure 1(A)). Before therapy, a mean VAS score of 5.1 was reached. This value decreased to a minimum of 4.2 (after 12 weeks) and remained at this lowered level for the rest of the observation period. Interestingly, at the end of the study this pain relief was evaluated as even higher when the patients were asked to retrospectively estimate their pain progression (Figure 1(B)). They indicated a higher pain value before therapy (6.0 VAS points) compared to when asked on-site. This pain reduction was experienced by 87% of all patients, whereby 18% had a transient and 69% a lasting effect (Figure 1(C)). This long-lasting pain reduction was confirmed by the more objective pressure point measurements (dolorimetry). An increase in applicable pressure representing a decrease in personal pain sensitivity was observed after radon spa therapy over the complete observation period (Figure 1(D)). This applied for the mean of all trigger points as well as for the one located in the most affected area. Furthermore, 81% of all patients stated that they would repeat this therapy and 96% said that they would recommend radon spa therapy to others.
Immune monitoring

Major immune cells

The composition of the major immune cells was hardly affected by the radon spa therapy (Figure 2). Nevertheless, a slight but long-lasting increase in T cells and monocytes was observed. The T cells increased on average from 22.5% to 24.2% (Figure 2(A)) and the monocytes from 6.2% to 6.9% (Figure 2(D)). Further on, neutrophils temporarily decreased slightly from an average of 55.6% to 53.2% shortly after therapy (Figure 2(G)). Simultaneously, eosinophils increased from 3.9% to 4.2% (Figure 2(H)).

Greater impact was determined on the DCs that circulate the periphery in very small numbers. They were directly determined as the plasmacytoid DC (pDC) and myeloid DC (mDC) subsets. The latter showed an increase of 21.2% (from 0.26% to 0.31% of all leukocytes, Figure 2(E)) whereas the number of pDC rose by 12.8% (from 0.15% to 0.17%; Figure 2(F)). Interestingly, a subdivision of mDCs into type I (mDC-i) and II (mDC-II) revealed that only the mDC-I increased (of 21.8%, from 0.25% to 0.30%, not shown) and not the mDC-II. Collectively, the DCs temporarily increased from 0.41% to 0.48%, which equals an increase of 18.2%. No effects were detected on B or NK cells (Figure 2(B) and (C)).

Immune cell subsets

The CD4+ T helper cells (T_H; Figure 3(A)) and most of their subsets, including T_H1 (Figure 3(B)), T_H2 (Figure 3(C)), and T_H17 (not shown), did not show any alterations. The same was true for a functional distinction into naïve, effector, effector memory, and central memory T_H (data not shown).

However, we identified a significant increase in T regulatory cells (T_REG) from 7.2% to 7.4% (in relation to T_H cells) shortly after therapy for up to 12 weeks (Figure 3(D)). This increase was more prominent when evaluated in relation to all cells as the number of T cells rose in general with a total increase of 12.7% (week 6: from 1.10% to 1.26%; not shown) to 23.6% (week 12: from 1.10% to 1.36%; not shown).

Considering the CD8+ cytotoxic T cells (T_C), we found a small but significant decrease at later time points (Figure 3(E)) and shifts within the naïve, effector, and memory subsets (Figure 3(F–I)). Collectively, a shift from the naïve and central memory T_C to the effector and effector memory T_C was identified. This shift paralleled the general decrease in T_C and started in week 12. The naïve T_C decreased by 7.8% from 24.4% to 22.5% (related to all T_C; Figure 3(F)) and the number of central memory T_C dropped strongly by 49.0% from 7.7% to 3.9% (Figure 3(H)). On the other hand, effector and effector memory cells gained about 8.6% (Figure 3(G): from 40.4% to 43.9%) and 8.0% (Figure 3(I): from 27.5% to 29.7%) respectively.

Furthermore, a shift within the three NK cell subsets that were determined by their CD56 and CD16 co-expression was revealed. A small but significant increase in the main cytotoxic NK subset (CD56hi/CD16hi; termed NK1) shortly after therapy (Figure 3(J)) from 91.3% to 92.5% related to all NK cells) was seen. Both other subsets decreased (Figure 3(K) and (L)). The smallest subset NK3 (CD56lo/CD16hi) had a local minimum shortly after therapy (decrease from 2.7% to 0.2%), but recovered afterwards (Figure 3(L)). In contrast, NK2 (CD56hi/CD16hi) dropped continuously but very slightly throughout the observation period (Figure 4(K): from 6.0% to 5.2%). These three subsets were also investigated for their co-expression of NK cell-specific markers such as NKG2A (CD159a), NKG2C (CD159c), NKG2D (CD314) and CD94, but no modulations by radon spa therapy were found.

Moreover, no alterations within the B cell or monocyte subsets were observed. Likewise, we did not find any relationships between the therapy and the frequency of circulating hematopoietic stem cells (not shown).

Figure 2. Minor modulations of the major immune cells by radon spa therapy. The T cells (A) and monocytes (D) were slightly but long-lastingly increased after radon spa therapy. The B cells (B) and NK cells (C) were not affected. Regulatory DCs, which were identified as mDCs (E) and pDCs (F), were temporarily increased shortly after therapy. Likewise, the neutrophils (G) and eosinophils (H) were affected shortly after therapy. Box and whisker plots. All statistical analyses were performed versus time point 0 (before therapy) using the paired t-test. *p < 0.05, **p < 0.01; ***p < 0.001.
Activation level of the immune cells

In addition to determining cell subset compositions, we were interested in the impact of radon spa therapy on the activation state of these circulating immune cells. Therefore, the expression of common activation markers such as CD38 (cyclic ADP ribose hydrolase), CD69 (very early activation antigen), CD80 (B7.1), CD86 (B7.2), and HLA-DR (MHC class II) was analyzed. The most prominent effects were found for CD69 and HLA-DR expression on lymphocytes (Figure 4). CD69 expression was strongly decreased on all lymphocytes with a local minimum between 6 and 12 weeks after the start of the radon spa therapy. Its expression level on T cells dropped by 34.0% (Figure 4(A): from 15.7% to 10.3%, related to T cells), on B cells by 35.5% (Figure 4(B): from 15.5% to 10.0%, related to B cells) and on NK cells by 45.8% (Figure 4(C): from 29.1% to 15.8%, related to NK cells). In all three cases, the expression of CD69 rose again at the end of the observation period, but was still lower than before therapy.

HLA-DR is a marker that is usually rare on T cells. Accordingly, with 2.7% only a small proportion of T cells were expressing it. Nevertheless, this expression level continuously increased to an expression of 3.9% after 6 weeks and 4.3% after 30 weeks (Figure 4(D)). This equaled an increase of 32% to 58% and was a long-lasting effect. The investigation of this expression on the different T-cell subsets revealed variations between TH and TC. Shortly after therapy, HLA-DR expression on TH rose from 2.2% to 3.9% (Figure 4(E)) and on TC from 2.7% to 4.3% (Figure 4(F)).
Before therapy, 3.7% of all T cells were already expressing HLA-DR, which was higher from the start (Figure 4(F)). Before therapy, 3.7% of all T cells were already expressing HLA-DR and this level rose even further to 5.9% after 6 weeks (increase of 59%) and 6.7% after 30 weeks (increase of 79%).

**Discussion and conclusions**

In the past, observations of long-lasting pain relief following radon spa therapy of patients with chronic painful degenerative diseases have been described [2,3]. This was also demonstrated in the multicenter IMuRa trial with approximately 680 patients. This study investigated the radon spa therapy commonly applied in various health spas in comparison with a control intervention for rheumatic outpatients and its results suggested beneficial analgesic effects of this therapy in rheumatic diseases for up to 9 months of post-intervention [4]. These observations were also confirmed by the explorative RAD-ON01 study with 100 patients presented here. Nevertheless, sufficient evidence of the beneficial effects of radon spa treatments has not yet been provided, mostly due to the poor quality of many of the studies performed [26].

Prior to execution of the RAD-ON01 study, we hypothesized that the low doses of alpha radiation emitted by radon might affect the immune system and thereby contribute to a reduction in or even resolution of chronic inflammation as the main cause of painful degenerative diseases. Since the blood functions as a means of transport for immune cells to reach their target tissue, one would expect that feasible immune modulation properties of radon could be detected not only in the inflamed tissues but also in the peripheral blood. We expected that these small doses of alpha radiation as applied in radon spa therapy induce small alterations to the immune status. Now, for the first time, the RAD-ON01 trial has demonstrated a modulation of the peripheral immune system through standard radon spa therapy (Figures 2–4). These modulations might contribute to the pain-relieving effects of the therapy, but conclusive proof of the underlying mechanisms still remains a challenge.

Neutrophils play a major role in inducing and maintaining inflammation. We found that the number of these innate immune cells temporarily decreased after radon spa therapy, which may indicate reduced tissue inflammation. Simultaneously, the number of eosinophils was elevated. Eosinophils are commonly viewed as nonspecific destructive effector cells, which play a major role in allergies and parasitic infections. However, it is now assumed that eosinophils have regulatory functions in tissue homeostasis and repair mechanisms [27,28]. One might speculate that an elevation of eosinophils could contribute to the restoration of tissue homeostasis in chronically inflamed tissues.

Furthermore, we found the DCs to be temporarily elevated. These highly efficient antigen-presenting cells circulate through the periphery constantly, capturing antigens and presenting them to T and B cells [29]. The DCs express various pattern recognition receptors (PRRs) for classification of the captured antigens and depending on this, their presentation is accompanied by either stimulatory or anti-inflammatory signals for T and B cells [29]. Consequently, either adaptive immune responses or immune tolerance are induced, making DCs important regulators of the immune system. The blood DCs are differentiated into mDCs and pDCs, and both types were temporarily increased after the radon spa therapy. The mDCs express many different PRRs responding to several stimuli [29] while the pDCs are more specialized in sensing nuclear acids and fostering pro-inflammatory responses [30]. A temporary increase in DCs, as observed in the RAD-ON01 study, may indicate a rise in the number of these effective regulators for active suppression or resolution of chronic inflammation and again for restoration of tissue homeostasis.

Regarding the main immune cells, we also found the number of T cells and monocytes to be slightly but long-lastingly increased. This also applied to their expression of HLA-DR, which belongs to the MHC class II complex and is generally expressed on professional antigen-presenting cells to show their captured antigens to T or B cells. Still, a few T cells express HLA-DR upon activation [31] and these cells have been found to capture and present autoantigens to other T cells and thus actively suppress them [32]. In contrast to T cells, nearly all monocytes express HLA-DR, but an elevated expression level has been linked to a better therapy outcome in severe systemic inflammatory diseases [33,34]. Thus, an up-regulation of HLA-DR on both cell types after radon exposure might actively contribute to resolving chronic inflammation.

We also detected modulations within the T-cell subsets. Interestingly, no effects on the composition of TH or its subsets were detected despite their central role in the coordination of innate and adaptive immune cells. Only the TREGs, which are able to directly suppress the activation of other immune cells and are thus highly efficient and potent immune regulators even when occurring at very low frequency, were modulated. They play a significant role in the prevention of autoimmune diseases [35]. Even tiny increases as observed here could evoke potent local or systemic immune suppression.

In contrast to TH, we detected a late decrease in TC and different shifts within its subsets from week 12 onwards. The main function of TC is the cytotoxic destruction of infected or degenerated host cells. Therefore, the MHC class I complexes, which are expressed by all nucleated host cells and continuously present intracellular peptides, are screened for foreign (e.g. viral) or degenerated molecules (e.g. tumor proteins). As these MHC class I molecules are expressed by nearly all host cells, defects in their recognition may cause extensive tissue damage. Indeed, a subgroup of CD57 TC has already been shown to be elevated in patients with RA [36]. However, the role of TC in autoimmune diseases is still unclear and controversial [37], and animal studies on CD8- and CD4-deficient mice have suggested that TC may instead have a regulatory or even protective function in arthritis [38]. In the RAD-ON01 study presented here, we observed that TC decreased in the peripheral blood following the radon treatment, but further subtyping revealed shifts within the TC subsets in the direction of effector subsets, suggesting an active contribution of TC.
To summarize, we observed a late decrease in naïve and central memory TC in favor of effector and effector memory TC. In general, the first two circulate through the body or reside in tissues waiting to encounter their specific antigen, but lack inflammatory and cytotoxic functions [39]. Then upon challenge they proliferate extensively and generate effector or effector memory cells, which then migrate into the target tissues for elimination of infected or degenerated cells accompanied by secretion of cytokines and chemokines [40]. If available, the response of memory cells is much faster than that of naïve T cells [39] and it was observed that the effector memory cells increased earlier than the effector cells. However, occurring after around 12 weeks, all these effects appeared relatively late after radiation exposure, indicating that this response was a consequence of a previous occasion which was directly caused by radiation.

Lastly, shifts within the subsets of the innate NK cells were found even though their total number remained unaffected. The main cytotoxic NK1 subset [41] increased shortly after radon spa therapy and simultaneously the CD56+/CD16 subset, which has not yet been functionally characterized, decreased. Unlike this early modulation, the CD56hi/CD16 NK2 subset, which has a regulatory function and primarily supports other immune cells by cytokine secretion (e.g. IFN-γ, TNF-α, GM-CSF, IL-10, IL-13) [42], decreased continuously. However, we did not find any effects of the radon spa therapy on the expression of activating (NKG2c) or suppressing (NKG2a) molecules by NK cells (not shown). Thus, the role of NK cells remains elusive. However, this innate immune response might pave the way for the delayed TC response.

In conclusion, the prospective and explorative RAD-ON01 study shows for the first time that immune modulations that may favor the attenuation of inflammation occur in the peripheral blood following radon spa therapy. One might speculate that radon and its secondary products might contribute to the restoration of balance in chronically dysregulated inflammatory tissues. The latter are a major cause of clinical symptoms such as pain or joint stiffness. Certain immune modulations only occurred short after the end of therapy. However, some effects, in particular shifts within the TC subsets, appeared late or remained for a long time, indicating secondary radiation effects. The deactivation of immune cells as observed in the reduced CD69 expression on T, B, and NK cells may be responsible for the long-term improvement in clinical symptoms that was reported by the majority of patients.

A recently published randomized, placebo-controlled intervention study showed that exercise both with and without low-dose radon hyperthermia balneo treatment affected bone metabolism and quality of life in a study population of an age group at risk of developing osteoporosis [43]. However, the patients in the therapy group had a slightly stronger reduction in the osteoclast-stimulating protein receptor activator of nuclear κB-ligand [43]. This study nicely illustrates the need for further randomized trials to investigate the effects of low doses of radon on the human body. As mentioned earlier, a RAD-ON02 study with a cross-over design is on the way. The authors have nothing to declare. This work was supported by the German Federal Ministry of Education and Research (BMBF) under Grant 02NUK017G (GREWIS), in part by the European Commission (European Network of Excellence, DoReMi) under Grant FP7-249689, by Landesamt für Gesundheit und Lebensmittelsicherheit Bayern (LGL), and by Bayerisches Staatsbad Bad Steben GmbH.

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