

Chapter 2

Bacterial Preparations

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Abstract From the era of Coley's toxin back in the beginning of the twentieth century, it was well known that certain acute bacterial infection might lead to the regression of malignant tumors in some cases. Many types of bacteria either in its crude form or with special preparation have been reported to possess immunotherapeutic activity against cancers. To date, experimental studies and clinical implications of those tumor immunotherapies have become more widely examined with more sophisticated methodology, utilizing activation of the two types of human immune system, i.e., innate and adaptive. In this chapter, various bacterial preparations that have been applied for tumor immunotherapy will be introduced, together with the new detailed mechanism of action of those two immune systems that have recently been elucidated. Of note, the efficacies of OK-432, a preparation derived from *streptococcus pyogenes*, are discussed by a tabulated data and individual patient data meta-analyses of randomized trials of adjuvant immunochemotherapy for lung and gastric cancers.

Keywords Bacterial preparation • Tumor immunity • Innate immune system • Adaptive immune system • Adjuvant immunochemotherapy clinical trials of OK-432

2.1 Everything Started from Coley's Toxin: Recent Findings Elucidating Mechanisms of Its Antitumor Activity

Spontaneous regressions of various types of cancer have been reported in the history of medical science. Regression is more commonly associated with groups of tumors like the embryonal tumors in children, breast cancer, chorioepithelioma,

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malignant melanoma, neuroblastoma, sarcomas, bladder cancer, skin cancer, and renal cancer [1, 2]. These phenomena are sometimes associated with infection, vaccine therapy, and incomplete surgical removal of the tumor [3, 4]. In line with those findings, presence of autologous cancer-specific antigens was enthusiastically pursued in melanoma and renal cancer patients in the 1970s and 1980s. Although scientifically intriguing, those studies could not have lead to any successful clinical implication [5, 6].

On the other hand, despite the assumption and fact that chronic infection may lead to cancer, it is presumed that acute infection, on the contrary, could have beneficial effects and often contributes to complete eradication of cancers even with a large tumor burden. In this regard, the use of microbial vaccines for immunotherapy is still being reexamined. This therapeutic concept is based on the early work of Coley, who reported infection-associated tumor regression over a century ago [7]. Inspired by the findings, he injected his first patients with vital *Streptococcus pyogenes*, a gram-positive organism causing erysipelas. By that attempt, although tumor shrinkage was observed, lethal systemic infections occurred. Thus, Coley modified his treatment regimen using a mixture of heat-inactivated *Streptococcus pyogenes* and *Serratia marcescens*. The inoculation of this bacterial vaccine, later known as “Coley’s toxin” (CT), marked the origin of modern immunotherapy, and thus Coley is also referred to as “father of cancer immunotherapy” [8].

Recent comprehensive discoveries have deepened more precise understanding of the immune system. Coley himself believed that the effect of his bacterial mixture was based on the release of toxins affecting tumor but sparing normal cells [9]. In fact, CT activates the innate as well as the adaptive immune system by binding toll-like (TLR) and other pattern recognition receptors. With regard to the bacterial nature of CT, this mixture contains unmethylated cytosine phosphodiester guanine complex (CpG), lipoteichoic acid, and lipopolysaccharide (LPS), acting agonistic with several TLRs [10]. Engagement of TLRs induces an inflammatory cascade resulting in cytokine secretion and immune cell activation [11]. This proinflammatory milieu together with high fever breaks the tumor-induced immune tolerance and changes it to an antitumor immunity [12–14]. However, Coley’s original hypothesis resting on an immune reaction against a “toxin” present in the microbial material that cross-reacts with and destroys the tumor cells falls more and more into oblivion and up to now has only partly been reexamined [7].

In the 1960s and 1970s, commercial CT preparations were tested on small patient cohorts. In these experiments, results were variable—presumably because of relatively short treatment courses. Also, most of the patients were immunocompromised due to prior or coadministered chemotherapy [15, 16]. Besides, plenty of immune mediators relevant for the inflammatory process were used as single agents in cancer immunotherapy [17–19]. But most of them failed to prove clinical efficiency.

Indeed, the benefit of CT treatment is supposed to be based on the chronological sequence of single immune mediators to induce an optimal antitumor immune response. These facts strengthen the usefulness of a comprehensive analysis regarding the therapeutic potential of the toxin, generated from the original protocol.

In 2005, a Canadian company (MBVax) started to produce CT and rekindle Coley's pioneer work. Since then, promising results for different tumor entities were obtained. These findings are an inducement for further investigations on the antitumor effect of CT.

Analysis of the potential of CT to affect tumor cell growth both in vivo and in vitro was implemented, taking advantage of its intrinsic immunestimulatory properties [20]. Examination of a direct impact on cell growth, proliferation, and viability was also performed. Of note, proapoptotic molecules in tumor target cells increased upon CT treatment [21]. In vivo, repetitive local CT applications effectively controlled tumor growth by stimulating immune responses.

The main objective of this chapter is to examine whether a purely microbial-based approach, or in combination with additional chemo- or targeted therapies, can cure non-immunogenic tumors. Picking up the historical idea of using Coley's toxin (CT), a complex mixture of gram-positive and gram-negative bacterial components as an active antineoplastic agent could be centered as a basis of immunotherapy or immunochemotherapy for cancers using bacterial preparations.

For potential clinical application of microbial-based vaccines, several requirements need to be complied. These include (I) reducing non-specific toxicity to normal cells, (II) preserving antitumor and tolerance-breaking immunestimulatory potential, and (III) applying a standardized treatment protocol. As for the latter, no such standardization was done in the past. Hence, Coley's work came under criticism, because at that time, 13 different preparations and various administration routes (i.v., i.m., and i.t.) existed, and some of these were more effective than others [9, 22]. This may explain why Coley's results could not be reproduced by others. In order to overcome this obstacle, CT should have been designed under constant, standardized conditions according to the original protocol.

Results of recent studies have demonstrated that this preparation acts as a potent antitumor and immune-activating agent. In a series of in vitro experiments, induction of cell death in tumor target cells was observed despite different susceptibility of cancer cells toward CT was demonstrated. In one of those studies, while AsPC-1 cells responded with substantial cell death, other cell lines were less affected. As central mechanisms of CT-induced growth alteration, an upregulation of p21waf gene expression and loss of G2/M phases, both indicative for cell cycle arrest, were reported [21]. In line with the established capacity of bacteria to induce apoptosis as well as necrosis in target cells, both kinds of cell death were observed. Proteins (i.e., LPS, Flagellin) delivered by *S. marcescens* may thus have preferentially induced necrosis, while factors provided by *S. pyogenes* (i.e., streptokinase, streptolysin, and lipoteichoic acid) led to apoptosis [7, 23, 24]. Accordingly, caspase 3/7 activation and DNA fragmentation were also detected in CT-treated tumor cells.

In addition to the capacity of directly compromising tumor viability, CT was also described as being a strong immune stimulator [9, 25–27]. The bacterial DNA (CpG ODN) present in this complex mixture may here be one of the best-known immune-activating candidates. CpG ODNs have been found to improve antigen-presenting cell functions and boost humoral as well as cellular Th1-directed immune responses. They have shown promising results as adjuvants for vaccines

and in combination with radio- or immunotherapy [10, 12, 28]. Several CpG ODN-based agents were already included into clinical trials for exploring their safety and efficacy in hematological and solid cancers [28–30] [<http://www.clinicaltrials.org/>]. The underlying mechanism is due to activating TLRs, the most important innate immune receptors [12–14]. TLR signaling in immune cells is crucial for regulating innate and adaptive immune responses, such as DC maturation and antigen presentation as well as CD8+ T cell toxicity [31, 32]. 6#CT-stimulated leukocytes from healthy donors could be effectively activated and responded with upregulation of TLR 2, 5, and 9. Likewise, CD25 expression was significantly and sustainably induced in these short-time-mixed leukocyte cultures, suggesting a stimulation of γ/δ T cells [33]. Besides, secretion of Th1 and other proinflammatory cytokines (e.g., IFN- γ , IL12, and TNF- α) by immune cells belonging to both the innate and adaptive arm can also be anticipated. Hence, this mixture of TLR agonists likely stimulates a complex cascade, each of which plays a unique and vital role in orchestrating immune responses [34]. A boost of antitumor effects with a massive decrease in tumor cell numbers was also observed together with CT. That boosted antitumor effects could rather be dependent from tumor-specific than from the allotransplanted lymphocyte. T3M4 and BxPC-3 could be effectively killed by CT and leukocytes. However, comparable results were not obtained for AsPC-1 cells, which had been shown to be highly susceptible toward CT-mediated lysis alone. This can be attributed to a kind of tumor-escape mechanism. These cells probably secrete immunosuppressive factors (IL10, TGF- β), thereby preventing leukocyte stimulation and immune-mediated lysis.

In a subsequent syngeneic *in vivo* tumor model, CTs' potential to impact solid tumors was examined. Efficacy for a non-immunogenic tumor was examined although it is clearly established that low immunogenic tumors respond worse than their immunogenic counterpart [9]. As a result, in immunocompetent mice-bearing syngeneic tumor, strong oncopathic effects were demonstrated after repetitive challenge of CT. Of particular interest was the finding that maximal tumor growth control was obtained after six injections. Increasing the number of injections did not further boost therapeutic responses.

Hence, CT may thus be best combined with other (antineoplastic) drugs rather than used as a single agent. However, before further exploring such combinatorial approaches, possible intolerable toxic side effects (e.g., cardiac, gastrointestinal, and hematological toxicity, anorexia, neuropathy, arthralgia, and myalgia) have to be excluded or at least minimized. Additional to identifying the optimal nontoxic dose, a proper application route (i.e., systemic versus local), an appropriate and feasible time schedule (simultaneous versus consecutive therapy), and potential synergistic or antagonistic effects of selected combinations have to be evaluated. All in all, CT might still be worth being employed for cancer immunotherapy due to its direct antitumoral as well as indirect immunostimulatory capacity.

2.2 Various Bacterial Preparations for Cancer Therapy and Two Different System of Antitumor Activity

Role of bacterial infection involving immunotherapy of cancers has been investigated and reported. The most prominent agent is *Mycobacterium bovis* bacillus Calmette-Guérin (BCG). Molecular and cellular mechanisms of BCG involved in immunotherapy of cancers have been discovered in the past decades, and the details of the study will be precisely described in Chap. 4 of this book.

Although BCG has been widely used in experimental and clinical immunotherapy, in view of the problems associated with the use of a viable organism, more defined, nonliving mycobacterial products need to be examined. In this regard, various bacterial preparations have been examined for its efficacy as immunotherapeutic agents in experimental animals and in clinical studies (Table 2.1).

As listed on Table 2.1, infection of many types of bacteria, either in its crude form or with special preparation, has been reported to have immunotherapeutic activity against various cancers [35–69]. However, despite strenuous efforts of physicians and investigators, very few have actually been examined in clinical trials for humans [37, 38, 41, 44, 47, 50, 55, 56, 59, 62, 68, 69], and furthermore, most of those clinical studies so far could not have shown definitive effect of immunotherapy with bacterial preparation to date. In reality, therefore, the inherent inefficiency of the immune system has given rise to numerous and highly expensive cytotoxic cancer therapies over the past few decades with almost no real benefits translated to the patient such as a cure or decreasing the chances of patient dying from cancer [70].

The human immune system can be broadly divided into two parts, the innate and the adaptive. The evolutionarily older innate immune system reacts within minutes after the invading pathogens are encountered. The adaptive system, which employs evolutionarily younger and more customized tools, takes longer time from days to weeks to generate specialized antibodies and T cells to attack threats [71]. The innate system consists of natural anatomical barriers, such as skin and mucous membranes, and physiological barriers like elevation of temperature and acid in the stomach to digest harmful bacteria as well as the cells of first defense. Innate immunity is effective against a variety of infectious agents that have common features recognized by phagocytic cells but has no immunological memory against previous exposure and is antigen independent [72].

The innate system is composed mainly of natural killer (NK) cells, polymorphonucleocytes (PMN) and macrophages, and is most directly involved in tumor immunology. These cells also participate in the adaptive response and form an important and vital bridge between the two arms of the immune system [71]. It recognizes nonself molecules according to a specific pattern. Another feature of the innate immune system is the complement, a group of inactive proteins in the blood which are activated in the presence of pathogens and nonself cells and cause cell lysis [73]. Attenuated inactivated bacteria as nonspecific tumor immunotherapeutic agents have been investigated for centuries [74]. Among those, *Clostridium perfringens*, *Streptococcus pyogenes*, and *Mycobacterium bovis* (will be described

Table 2.1 Bacterial preparations investigated for immunotherapy of cancers (excluding BCG)

Bacteria	Target cancer	Model	References
<i>Corynebacterium parvum</i>	Mastocytoma	Mouse	[35]
	Melanoma	Mouse	[36]
	Lung cancer	Human	[37]
	AML	Human	[38]
<i>Nocardia rubra</i>	Fibrosarcoma	Rat	[39]
	Melanoma	Mouse	[40]
	Lung cancer	Human	[41]
<i>Serratia marcescens</i>	Fibrosarcoma	Mouse	[42]
	Sarcoma	Mouse	[43]
	Malignant astrocytoma	Human	[44]
<i>Lactobacillus casei</i>	Lung cancer	Mouse	[45]
	Fibrosarcoma	Mouse	[46]
	Cervical cancer	Human	[47]
<i>Staphylococcus aureus</i>	Breast cancer	Mouse	[48]
	Ehrlich ascites tumor	Mouse	[49]
	Malignant tumors	Human	[50]
<i>Listeria monocytogenes</i>	Breast cancer	Mouse	[51]
	Melanoma	Mouse	[52]
	Review		[53]
<i>Mycobacterium smegmatis</i>	Bladder cancer	Mouse	[54]
	Melanoma	Human	[55]
	Lung cancer	Human	[56]
<i>Mycobacterium vaccae</i>	Non-small cell lung cancer	Human	[57]
	Melanoma	Human	[58]
	Renal cancer	Human	[59]
<i>Salmonella typhimurium</i>	Plasmacytoma	Mouse	[60]
	Review		[61]
	Pancreatic cancer	Human	[62]
<i>Clostridium perfringens</i>	Sarcoma	Mouse	[63]
	Leukemia	Mouse	[64]
<i>Clostridium novyi</i>	Colon cancer	Mouse	[65]
	Colon cancer	Mouse	[66]
<i>Streptococcus pyogenes</i>	Leukemia	Mouse	[67]
	Sarcoma	Mouse	[68]
	Mammary cancer	Rat	[69]
	Lung cancer	Human	[70]
	Uterine cervical cancer	Human	[71]

in Chap. 4) were considered to be the most active agents to induce tumor regressions for human tumors. Since we have already mentioned about the efficacy of Coley's vaccine in the previous paragraph, the other prominent nonspecific immunopotentiator OK-432, derived from *Streptococcus pyogenes*, will be introduced and discussed in the next paragraph [67–69].

The adaptive or acquired immunity is antigen specific, slower, and possesses immune memory against future attacks. The adaptive response follows the innate response and is dependent on specific recognition of antigen by antigen receptors present on the cell surface. The two types of adaptive immunity are cell-mediated immunity and humoral immunity. T lymphocytes are responsible for cell-mediated immunity and B lymphocytes for humoral immunity. B cells play a role in destroying tumor cells by complement-mediated lysis and facilitating antibody-dependent cell-mediated cytotoxicity [75]. The cytotoxic T cells (CTC) and the natural killer T (NKT) cells are two important T cells which are involved in lysis of tumor cells. The CTC kill and target cells with MHC–antigen complex on the cell wall, while the NKT cells actively search and kill tumor cells and play a crucial role in preventing metastasis of cancer. Affected cells which do not display MHC–antigen complex are targeted by the NK cells [76, 77]. Linking the innate and adaptive immune systems are dendritic cells that hugely play an important role in restraining cancer. Dendritic cells migrate and are found patrolling below and within the epidermis and mucous membranes in the mouth, nose, ear, and colon. These cells produce antigens from ingested pathogens and cell debris, carry them to the lymph nodes, and display them on their surfaces to T cells. Thus, the T cells and B cells are stimulated to customize their immune attacks [73, 74] (Fig. 2.1). Briefly,

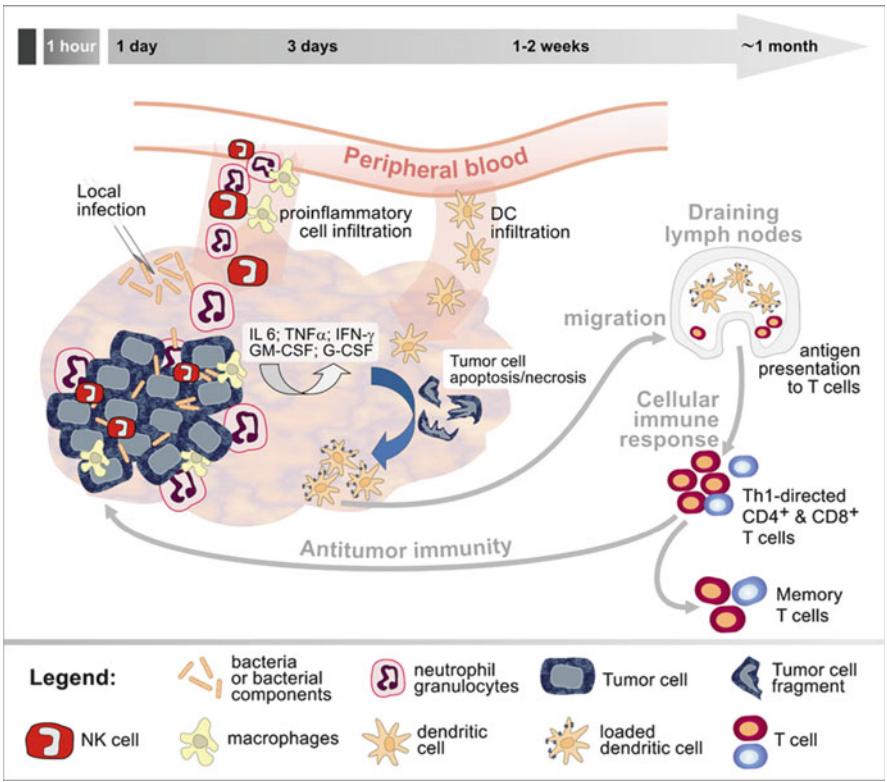


Fig. 2.1 Schematic representation of bacterial immunotherapy's mode of action

intratumoral bacteria or bacterial components sensitized by the innate immune cells like NK cells, macrophages, and neutrophils, followed by secretion of proinflammatory cytokines and chemokines, attracting immature dendritic cells (DC) into the focus of infection. DCs take up bacterial material together with tumor fragments, mature while migrating to draining lymph nodes, where DCs present tumor antigens in addition to bacterial antigens to T cells. Those activated T cells infiltrate the tumor microenvironment and kill tumor cells, whereby the patient benefits from an active and powered immune response that fights the infection as well as the cancer. To be functionally active, DCs need certain danger signals to activate them, such as the pathogen-associated molecular patterns (PAMPs) that are present on bacteria and viruses but are absent on the cancer cells; that is clearly a situation of dual advantage [12]. Long-lasting antitumor immunity having the potential to control micro metastasis will be established when part of these T cells becomes memory cells.

2.3 OK-432, an Immunopotentiator Derived from *Streptococcus pyogenes*: Innate and Adaptive Function

OK-432, a preparation derived from *Streptococcus pyogenes* has been used for the treatment of curatively resected non-small cell cancers, and relatively favorable responses have been reported [68]. However, various clinical trials performed to assess the benefit of immunochemotherapy including OK-432 have not shown a significant benefit on survival of the cancer patients. Sakamoto et al. have collected results from randomized trial evaluating the superior effect of immunochemotherapy over chemotherapy alone. Meta-analysis to review all the relevant trials is considered to give rise to the best methods with a reasonable chance of detecting small, but humanly worthwhile, clinical benefits for lung cancer patients. In their meta-analysis, 1520 patients enrolled in 11 randomized clinical trials were examined comparing standard chemotherapy with the immunochemotherapy using the same chemotherapy regimen plus OK-432, the *Streptococcus pyogenes* preparation [78]. The 5-year survival rate was 51.2 % in the in the immunochemotherapy group versus 43.7 % in the chemotherapy-alone group. The odds ratio (OR) for 5-year overall survival was 0.70 (95 % CI = 0.56-0.87, $p = 0.001$) (Fig. 2.2).

In other meta-analysis, efficacy of OK-432 immunochemotherapy over chemotherapy-alone treatment was examined in 1522 patients enrolled in six clinical trials for curatively resected gastric cancer [79]. By this meta-analysis, the 3-year overall survival rate was 67.5 % in the immunochemotherapy group versus 62.6 % in the chemotherapy-alone group (OR; 0.81, 95 % CI; 0.65–0.99, $p = 0.044$) showing borderline effect of immunochemotherapy (Fig. 2.3).

Although the above mentioned two reports seem to have demonstrated the definite benefits of the addition of OK-432 for conventional chemotherapy, the possibility of bias due to several prognostic factors could not be excluded, since the

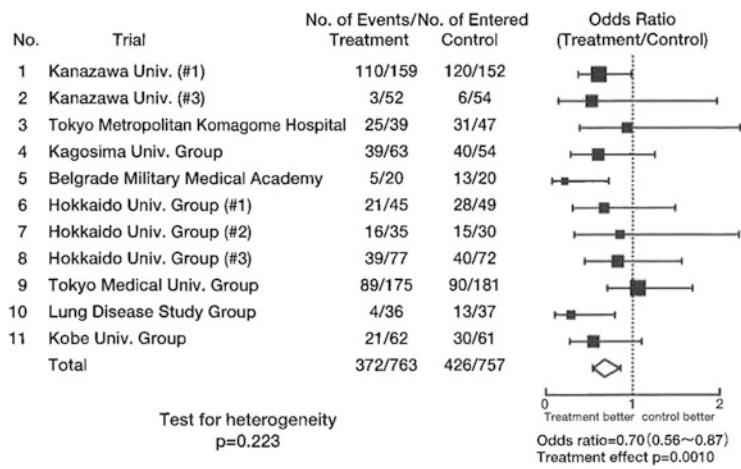


Fig. 2.2 Survival odds ratios of non-small cell lung cancer patients in individual trials and overall. The overall test for treatment effect was significant ($p = 0.001$)

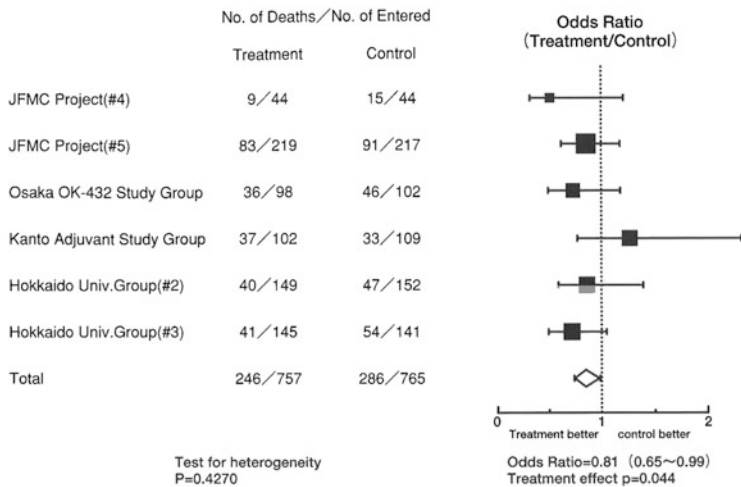


Fig. 2.3 Survival ratios of curatively resected gastric cancer patients in individual trials and overall. The overall test for treatment effect showed borderline significant benefit of the addition of OK-432 ($p = 0.044$)

study was performed based on the tabulated data from a meta-analysis of the randomized trials.

In recent years, new aspects of OK-432 treatment have been investigated since the beginning of the twenty-first century, and multiple lines of evidence for the effects of OK-432 have been reported. With the increased attention on OK-432 therapy, a detailed reevaluation of the results of cancer therapy using OK-432 in previous clinical trials was determined to be important. In this regard, collection of

the individual patients' data that were enrolled in eligible randomized trials and reexamination of the precise effects of immunotherapy using OK-432 in an adjuvant setting should be imperative. Since the current standard of treatment for patients with stage I or II gastric cancer after curative resection does not necessarily involve adjuvant chemotherapy, stage III or IV curatively resected gastric cancers have become the new target of the analysis. This reanalysis was important in order to clarify the immunological effects of OK-432, which has become widely utilized as a new immunotherapy and vaccine therapy for various cancers.

One thousand nine hundred and fifteen individual patients' data from 14 clinical trials were provided, and robust results showing a significant effect of OK-432 for locally advanced stage (III and IV) gastric cancers was confirmed and published [80].

Turning into the twenty-first century, more meticulous and diverse modes of action for OK-432 were investigated. Okamoto et al. precisely investigated the components of OK-432 and found that a lipoteichoic acid-related molecule is an active component of OK-432 stimulating TLR4/MD2 complex and Λ -interferon production [81]. They have also reported that DC maturation and Th-1 cytokine stimulation by OK-432 are highly reliant on the expression of TLR4 and MD2 genes [82]. They have also shown the TLR4 expression-dependent anticancer immunity both in an OK-432-immunotherapy model using the TLR4-deficient mouse and in the OK-432 treatment of patients with head and neck cancer [83]. Taken together, these findings strongly suggest that the expression of TLR4, probably with MD2 on ascites cells could essentially be required for TNF induction in order to obtain positive clinical responses for locoregional immunotherapy with OK-432 to malignant ascites from gastric cancer.

Another aspect of antitumor effect of OK-432 has also been highlighted. Results from a trial in which the cancer vaccine NY-ESO-1 was mixed with OK-432 and Montanide® also suggested intervention in the immune tolerance system intercalated by PD-1 on CD4 lymphocytes [84]. Phase I clinical trials utilizing OK-432 plus HER2/neu and NY-ESO-1 have been started for the clinical implication against esophageal, lung, stomach, breast, and ovarian cancers [85].

2.4 Summary

Bacteria, either used as direct anticancer agent or as a vehicle for cytotoxic agents, mediate strong pro-inflammatory reactions that have beneficial effects for tumor therapy. In an acute phase, bacteria massively activate the immune system initiating an unspecific, often neutrophil-directed reaction that is followed by a Th1- or cytotoxic T cell-directed cellular response, eventually providing long-term protective immunity.

Bacteria and their components, mainly defined as TLR ligands or PAMPs, can be safely applied in humans with limited adverse side effects and are thus established

in the clinic as immunestimulatory adjuvants. Combination therapies are also being investigated for potential future applications.

Those recent findings have been able to provide a ready basis for further expanding the concept of cancer immunotherapy for the clinical setting.

Conflict of Interest JS have been receiving honorarium from Takeda Pharmaceutical Inc. and from Merck Serono Co. Ltd.

Acknowledgment Collection of the materials and information for this review were supported, in part, by Epidemiological and Clinical Research Information Network (ECRIN).

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Immunotherapy of Cancer

An Innovative Treatment Comes of Age

Yamaguchi, Y. (Ed.)

2016, IX, 358 p. 46 illus., 38 illus. in color., Hardcover

ISBN: 978-4-431-55030-3